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Effects of halogenated substances on testicular cells – Risk assessment and DNA damage

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Abstract

This study investigates four halogenated substances; three polyfluorinated compounds and one brominated substance. The polyfluorinated compounds, namely 6:2 fluorotelomer alcohol (6:2 FTOH), 8:2 fluorotelomer alcohol (8:2 FTOH) and perfluorooctanoic acid (PFOA), are widespread anthropogenic compounds, used in a variety of consumer products due to their water-, grease- and stain-repellent property. Levels of PFOA in human blood are typically around 5 ppb (0.01 μM), while FTOHs so far not have been measured in human samples due to technical difficulties. One of the main effect of PFOA in laboratory animals and in *in vitro* experiments is an induction of peroxisomal proliferation, leading to increased oxidation of fat, changed expression of a variety of genes, and increased cell proliferation, possibly inducing cancer development, both in the liver and in the pancreas and testicle. PFOA is also a reproductive toxicant, changes membrane architecture, and inhibits the effluent transporter P-gp. Several of the effects of the FTOHs are similar to those observed after PFOA exposure and several of the genes that show altered expression after PFOA exposure are also changed after exposure to 8:2 FTOH. Effects of 8:2 FTOH exposure include hepatocellular necrosis and peroxisomal proliferation, as well as detrimental effects on development in mice. Both 6:2 and 8:2 FTOH have been shown to act as xenoestrogens *in vitro*.

We evaluated the effect of 6:2, 8:2 FTOH and PFOA on testicular cells from Wistar rats. Testicular cells were exposed *in vitro* to concentrations up to 300 μM for one hour. No signs of cytotoxicity were observed. The level of single strand breaks, abasic sites and oxidized purines was not increased, either. Whether the expression of the breast cancer resistant protein (Bcrp1) was altered, remains unsure due to high variability between experimental runs. One major weakness in these experiments is the use of a relatively short exposure time, making it very difficult to draw conclusions. Since the tested concentrations are many magnitudes higher than exposure in the general population, the data have nonetheless predictive value. Taken together, the results suggest that the tested PFCs do not exhibit testicular toxicity.

The brominated compound 1,2-dibromo-3-chloropropane (DBCP) is known to induce permanent or temporary infertility in men, in addition to being a renal toxicant. It also induced DNA damage and acts as a clastogen and mutagen, inducing cancer development. The DNA inducing effect of DBCP on three testicular cell types was evaluated; Sertoli cells and other somatic cells seem to be the most sensitive cell type, spermatogonia the least sensitive, and spermatids seem to have a medium sensitivity. However whether the results obtained in experiments with somatic cells are correct is quite uncertain due to prolonged perincubation

time. The repair capacity in spermatogonia and spermatids was also examined; spermatogonia were found to repair DNA damage induced by DBCP somehow faster than spermatids. Taken together, the results suggest that it may be the supporting cells like Sertoli cells that get heaviest damaged by DBCP. The damage in these cells can then lead to impaired germ cell development by interfering with the supply of nutrition, testosterone and other supporting functions. Spermatogonia appear to be least sensitive and repair the induced DNA damage relatively effectively. Since these cells are located outside of the blood-testis barrier these efficient defense mechanisms are very meaningful.

Contents

Abbreviations

DBCP	Dibromochloropropane
PFC	Polyfluorinated compounds
PFOA	Perfluorooctanoic acid
FTOH	Fluorotelomeralcohol
uFTOH	α-β unsaturated fluorotelomer alcohol
FTAL	Fluorotelomer aldehyd
FTUAL	α-β unsaturated aldehyd
FTA	Fluorotelomer acid
FTUA	α-β unsaturated acid
BCRP1	Breast cancer resistance protein
ABC-transporter	ATP-binding cassette transporters
MDR	Multidrug resistance
MRP	Multi drug resistance protein
P-gp	P-glycoprotein
OAT	Organic anion transporter
BTB	Blood-testis barrier
BBB	Blood-brain barrier
GSH	Gluthatione
GST	Gluthathione S-transferase
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
RNA	Ribonucleid acid
cDNA	Complementary DNA
PP	Peroxisomal proliferator
ROS	Reactive oxygen species
LD50	Lethal dose for 50% of tested animals
Fpg	Formamidopyrimidine-DNA-glycolase
ROS	Reactive oxygen species
8-oxo-G	7,8-dihydro-8-oxoguanine
FSH	Follicle stimulating hormone
LH	Lutenizing hormone
ppm	Parts per million
ppb	Parts per billion
ppt	Parts per trillion

Aim of study

The number of man-made substances is ever increasing. Many of these substances become widespread in the environment, but the effects these have on human health are not always known. Risk assessment is a method for evaluating the detrimental effects of chemical substances. To be able to perform a risk assessment, the intrinsic effects of a component have to be known, as well as "safe" levels and exposure scenarios. This study attempts to contribute to all these parts of the risk assessment by performing a literature study to evaluate the existing information on toxicity of the components, as well as identify areas where further research is needed. A component of this part is also the assessment of human exposure. However, this has proven to be difficult due to lack of data concerning several of the examined components. Further on, this study tries to fill some of the knowledge gaps by performing experiments. Four substances where chosen in this study: 6:2 fluorotelomer alcohol (6:2 FTOH), 8:2 fluorotelomer alcohol (8:2 FTOH), perfluorooctanoic acid (PFOA), and 1,2-dibromo-3-chloroporoane (DBCP). No information concerning the testicular toxicity of the polyfluorinated compounds (PFCs) could be found, so this was identified as one of the main areas where further research was needed. DBCP on the other hand, was used as a positive control in the experiments with the PFCs, since it is known to be a testicular toxicant. Due to a difference in response found in different testicular cell types after DBCP exposure, this compound was studied in more detail.

The overall aim of the study is thus to contribute to a risk assessment of the four halogenated compounds by summing up existing information, but also creating new information. To achieve this, five goals were identified:

1. Perform a literature study on the selected halogenated compounds.
2. Evaluate the toxicity of three selected fluorinated compounds on adult testicular cells regarding their
    a. Cytotoxicity
    b. DNA damaging effect
    c. Gene expression alterations
3. Evaluate a simplified method for isolation and purification of spermatogonia from prepubertal rats.
4. Investigate the difference in susceptibility of various testicular cell populations towards DNA damaging effects of DBCP.
5. Assess a possible difference in repair efficiency in spermatids and spermatogonia after exposure to DBCP.

1. Introduction and background

An undisturbed reproductive process is not only important for the survival of the species, but it is also fairly important for each person individually. In Western countries, one out of seven couples is experiencing fertility problems, of which at least half have unknown causes (Olive and Cuzin, 2005). Many factors can intervene with the reproductive process and lead to reduced fertility; examples are genetic disorders, lifestyle factors, disease, and exposure to chemicals or radiation. One of the first references to a chemical interfering with reproduction is made by Aulus Gellius (ca 125-180 A.D.), cited in Robert Burtons Anatomy of Melancholy: "If a drunken man get a child it will never likely have a good brain" (Burton, 2001). Negative effects in both males and females are known, and yet research on effects of chemical agents has focused on female reproduction until the 1990s. One of the reasons for increased focus on effects in males is the observed increase in testicular dysfunction, anomalies and cancer, as well as decreased semen quality during the recent 60 years. This is believed to be due to environmental factors as well as lifestyle factors. The reduced male fertility can be due to a number of disturbances in the testicle; examples being the cell death of either the supporting cells or the germ cells, disturbance of hormonal balance leading to reduced sperm production, or DNA damage in germ cells, leading to apoptosis of the cell, inferior sperm quality possibly leading to reduced fertility and in severe cases to malformations and genetic defects in offspring.

In this study, two classes of compounds are surveyed: a well known reproductive toxicant, dibromochloropropane (DBCP), and a class of fairly "new" chemicals, polyfluorinated compounds (PFCs). DBCP is chosen to study the mechanism of testicular toxicity and as a positive control for experiments with the PFCs. We are especially interested in determining which period of germ cell development is most sensitive to lesions induced by DBCP. The polyfluorinated compounds are chosen because they are found in blood samples at quite high concentrations and are shown to induce reproductive defects in offspring following *in vivo* exposure, but have not been studied regarding their testicular toxicity. All compounds are evaluated regarding their cytotoxicity to testicular cells, as well as their DNA damaging effect. The polyfluorinated substances are also evaluated concerning their effect on gene expression of an efflux transporter pump, namely the breast cancer resistance protein (Bcrp1/Abcg2).

The introduction will therefore first give a summary of the available literature on the four selected halogenated compounds. Sources, toxicity and epidemiology of the surveyed compounds will be described. This is followed by an overview of the structure of the male reproductive system and different testicular cells and their functions, as well as DNA damage. Also, the function of the breast cancer resistance protein and other effluent transporters will be described.

1.1 Polyfluorinated compounds (PFC) – a literature overview

Polyfluorinated compounds (PFCs) are entirely man-made compounds and have been used since the 1950s (Poulsen *et al.*, 2005). They consist of a carbon chain where some or all of the hydrogen atoms have been replaced by fluorine atoms. The carbon-fluor bond is the strongest of all covalent bounds and resists hydrolysis, photolysis, biodegradation, and metabolism (Guruge *et al.*, 2006). This makes PFCs highly stable and persistent in the environment. PFCs have different functional groups like sulphate, acid or alcohol, which make one end of the molecule hydrophilic. The other end, containing fluorosubstitued carbon atoms, is hydrophobic, resulting in the molecule being amphipathic. This property makes PFCs water-, grease- and oil-repellent and therefore useful in a range of consumer products. PFCs are used in textile surface treatments, paper coatings, lubricants, fire retardants, electronics, waxes, polish, aerospace, paint-additives, and non-stick coatings for cookware, and many other applications. They are released during the production of consumer products, as well as during use of products.

PFCs ultimately end up in the environment and are found in a diversity of environmental samples: air, water, wild animals and birds, and foodstuff (Smith, 2001; Kannan *et al.*, 2002; Martin *et al.*, 2004; Skutlarek *et al.*, 2006; Barber *et al.*, 2007; Ericson *et al.*, 2007). The three PFCs found at the highest concentrations in air samples in Northwest Europe are PFOA, 6:2 fluorotelomer alcohol (FTOH) and 8:2 FTOH (Barber *et al.*, 2007). Additionally, PFCs are measured in samples from humans, including blood (Taves, 1968; Olsen, 2007) and breast milk (So *et al.*, 2006).

Because of the highly stable bond between carbon and fluorine, PFCs were historically considered metabolically inert and non-toxic (Sargent and Seffl, 1970). However, during the 1990s, evidence has accumulated of biological activity of PFCs and their toxic effects such as peroxisome proliferation, increased lipid metabolizing enzyme activity, and induction of Leydig cell adenomas and pancreatic and hepatic tumors (Sohlenius *et al.*, 1994; Obourn *et al.*, 1997).

Many different PFCs have been produced, but due to the scope of this thesis, only three PFCs were selected for further analysis: 6:2-fluorotelomer alcohol (6:2 FTOH), 8:2-fluorotelomer alcohol (8:2 FTOH), and perfluorinated octanoic acid (PFOA).

1.1.1 Fluorotelomer alcohol (FTOH)

The fluorotelomer alcohols (FTOHs) nomenclature are based upon the number of perfluorinated carbons in relation to the number of hydrogenated carbons. 8:2 FTOH refers to the 1H,1H,2H,2H-perfluoro-1-decanol with 8 carbon atoms being fully fluorinated and two carbon atoms without fluor, while 6:2 FTOH is the abbreviation for 1H,1H,2H,2H-Perfluoro-1-octanol (figure 1).

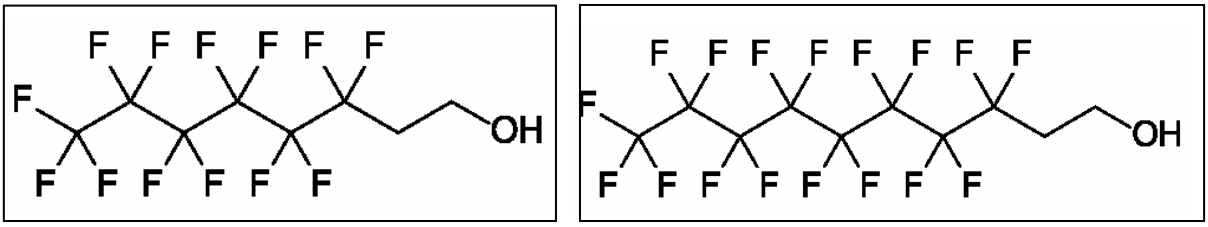

Figure 1: Structure formulas for 6:2 FTOH (left image) and 8:2 FTOH (right image)

There is hardly any information available on the toxicity of 6:2 FTOH so far, so this chapter will concentrate on 8:2 FTOH. However, the metabolism and toxicity of different FTOHs seem to be quite similar (Martin *et al.*, 2005), so most of the information presented here might be valid for 6:2 FTOH as well. 8:2 FTOH has been detected in bottlenose dolphins and arctic ringed seals (Houde *et al.*, 2006; Butt *et al.*, 2007).

Use and sources

FTOHs are used as intermediates in the manufacture of polymers for oil, soil and water repellent surface treatment applications, but also in the manufacture of paints, coatings, polymers, adhesives, waxes, polishes, electronics, and caulk. They get linked to the product via an ether-, urethane-, or ester-bond. When these bonds are not fully established during production, or when the bonds are degraded, FTOHs get released. The global annual production of FTOHs was estimated to 11-14 million kg in 2003 (Ellis *et al.*, 2003). DuPont reports a doubling of FTOH sales from 2000 to 2005 (Dupont, 2005). The FTOH used in the largest amount is 8:2 FTOH (Nabb *et al.*, 2007). Emitted FTOH can biodegrade to perfluorinated acids, 8:2 FTOH is suggested to degrade to a.o. PFOA, while 6:2 FTOH is expected to degrade to perfluorohexanoic acid (PFHxA) (Dinglasan *et al.*, 2004).

Toxicokinetics

In rats about 34-50% of 8:2 FTOH is absorbed after oral administration, but the absorption following dermal exposure is negligible (<1%). 8:2 FTOH has a very short half-life of less than 5 hours in rats (Fasano *et al.*, 2006). In the rat, 70% of 8:2 FTOH is excreted in the faeces, and metabolites are found in fat, liver, adrenals, and thyroid (Fasano *et al.*, 2006). The main fate of 8:2 FTOH is direct conjugation to form O-glucuronide and O-sulphate, which account for about 70% of metabolites (Martin *et al.*, 2005). Hagen *et al.* (1981) were the first to suggest metabolism of 8:2 FTOH to PFOA and 8:2-fluorotelomer acid (FTCA). Martin *et al.* (2005) found the initial step to be oxidation by P450-enzymes to 8-2 fluorotelomer aldehyde (FTAL). 8:2 FTAL is oxidized to 8:2-FTUAL or 8:2 FTA, which is further metabolized to 8:2 unsaturated acid (FTUA). 8:2 FTUAL can via several steps then form PFOA (figure 2). Many different sulphate-, glucoronide- and glutathione-conjugates may be formed (Nabb *et al.*, 2007). Nilsen *et al.* (2008) found that intraperitonally administered 8:2 FTOH was completely metabolized in Wistar rats within 24 hours . Similar results have been reported in exposed mice (Henderson and Smith, 2007). Nabb *et al.* (2007) examined the metabolism of 8:2 FTOH in rat, mouse, trout and human hepatocytes, microsomes and cytosols and found the elimination to be about three times faster in rodents than in humans and nine times faster than in trout. The amount of PFOA that was formed after 8:2 FTOH exposure was low: 0.47, 0.24, 0.02, and 0.02% of 8:2 FTOH was found as PFOA in mouse, rat, human and trout hepatocytes, respectively. These findings are in compliance with results of others, for instance Martin *et al.* (2005). Some of the intermediate metabolites have been reported to be more toxic than the perfluorocarbocylic acids (Phillips *et al.*, 2007); especially the FTUAL and FTUCA may react with cellular nucleophiles like nucleic acid (Martin *et al.*, 2005). FTOHs with different chain length have been shown to be metabolized to similar metabolites, with the only difference being the chain length of the metabolites (Martin *et al.*, 2005).

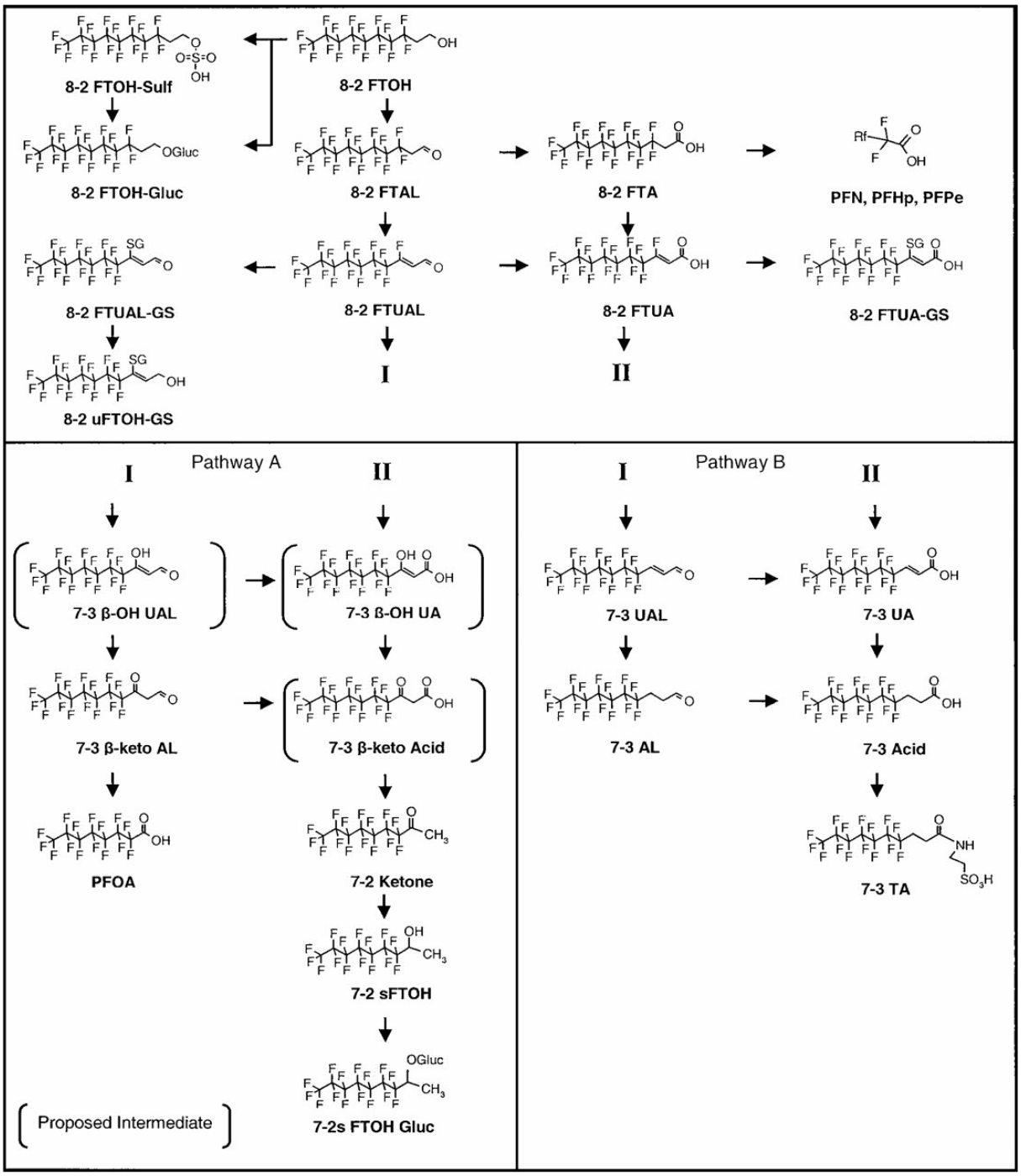

Figure 2: Metabolic pathway of 8:2 FTOH as suggested by Nabb *et al.* (2007). This figure incorporates pathways suggested by Martin *et al.* (2005) and Fasano *et al.* (2006). Metabolites shown in brackets are proposed intermediated (Martin *et al.*, 2005; Fasano *et al.*, 2006; Nabb *et al.*, 2007). FTOH: fluorotelomer alcohol; FTAL: fluorotelomer aldehyde; FTUAL: α-β unsaturated aldehyd; FTA: fluorotelomer acid; FTUA: α-β unsaturated acid; AL: aldehyde; UAL: unsaturated aldehyde; PFOA: perfluorinated octanoic acid.

Toxicity

The acute toxicity of 8:2 FTOH is low, with LD_{50}>2000 mg/kg bw in rats (Finlay, 2001). No LD_{50}-value for 6:2 FTOH could be found. In rat hepatocytes, concentrations of either 8:2 FTOH or 6:2 FTOH up to 200 μM did not influence cell viability as measured by the Trypan Blue exclusion test (Martin *et al.*, 2005). Exposure to 6:2 or 8:2 FTOH at concentrations up to $1.1x10^{-5}$M (11 μM) for 44 hours did not reduce cell viability in primary cultured *Tilapia* hepatocytes either (Liu *et al.*, 2007a). Stankowski (2001) tested the mutagenicity of 8:2 FTOH in the *Salmonelle typhimurium* strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* strain WP2*uvr*A. The compound was not found to be mutagenic in any of the strains with or without S9 (Stankowski, 2001).

In vivo exposure of rodents to 8:2 FTOH lead to similar signs of toxicity as with PFOA exposure: increased peroxisomal proliferation and hepatocellular necrosis (see chapter 1.1.2, toxicity of PFOA) (Ladics, 2003). Nilsen and co-workers found significant alteration of the expression of 105 genes following *in vivo* exposure to 8:2 FTOH (Nilsen *et al.*, 2008). Some of the altered genes overlap with genes altered by PFOA exposure; but alteration of genes not influenced by PFOA was also found. Other signs of toxicity observed in this study include hepatomegaly and induction of peroxisomal β-oxidation. Since no 8:2 FTOH could be detected in the liver 24 hours after the exposure, it is unclear whether the toxicity was caused by PFOA, 8:2 FTOH, or another metabolite (Nilsen *et al.*, 2008).

A mixture of different fluorotelomer alcohols (6:2 FTOH, 8:2 FTOH, 10:2-FTOH and 12:2-FTOH) was evaluated in an *in vivo* rat developmental and reproductive study by Mylchreest *et al.* (2005a). The authors estimated the NOAEL for reproductive effects to be 25 mg/kg/day and the NOAEL for developmental effects to be 200 mg/kg/day, with the most distinct effects being reduced litter size, and reduced weight gain, as well as fetal skeletal alterations (Mylchreest *et al.*, 2005a). The toxicity of different fluorotelomer alcohols is assumed to be similar, so the effect of this mixture is likely similar to the effect of one of the compounds at a time. This is confirmed by another study by Mylchreest *et al.* (2005b) where rats were exposed to 8:2 FTOH. The NOAEL for maternal and developmental toxicity was found to be 200 mg/kg/day (Mylchreest *et al.*, 2005b). Because signs of developmental toxicity did not occur prior to signs of maternal toxicity, 8:2 FTOH was not considered a selective developmental toxicant in rats. Henderson and Smith (2007) found that *in utero* exposure of mice to 8:2 FTOH lead to an increase of fetal PFOA concentrations. The observed accumulation in the foetus was explained by the pH gradient between the maternal and the fetal compartment. They also observed an increase in liver weight, mortality and

neural tube defects in neonates after administration of 30 mg/kg bw on gestational day 8 and classified 8:2 FTOH as a developmental toxicant in mice (Henderson and Smith, 2007).

Maras *et al.* (2006) found that both 6:2 FTOH and 8:2 FTOH act as xenoestrogens *in vitro*, with 6:2 FTOH having a slightly stronger potency. The authors report an effect on both cell proliferation and estrogenic gene expression. Ishibashi *et al.* (2007) observed an activation of the human estrogen receptor (hER) isoforms hERα and hERβ after incubation with 6:2 FTOH and 8:2 FTOH. A stronger induction was seen following 6:2 FTOH exposure compared to 8:2 FTOH (Ishibashi *et al.*, 2007). Liu *et al.* (2007a) came to a similar conclusion after detecting induction of vitellogenin in primary cultured *Tilapia* hepatocytes after 48 hours of treatment with 6:2 FTOH, suggesting an estrogenic activity of this compound. The authors interpreted this effect to be mediated by the estrogen receptor pathway. 8:2 FTOH did not induce vitellogenin. However, all these results are obtained in *in vitro* studies. In the *in vivo* reproductive study by Mylchreest *et al.* (2005a), no test-substance-related effect on estrous cycle parameters was observed.

Human exposure

FTOHs are absorbed after ingestion and inhalation, so possible exposure routes include air (gaseous FTOH as well as FTOH attached to particles) and contaminated food and drinking water. FTOHs have been detected in the North American atmosphere, in Europe and in the Arctic atmosphere (Stock *et al.*, 2004; Shoeib *et al.*, 2006; Jahnke *et al.*, 2007). Martin *et al.* (2005) estimated that the human exposure was between 0.2 and 2ng FTOH /kg bw/day. This was calculated from an outdoor concentration of FTOH of around 10-100 pg/m^3 (Martin *et al.*, 2005). This seems to be a relatively low estimate, considering that Barber *et al.* (2007) measured the concentration of 6:2 FTOH and 8:2 FTOH in the air to be 11-243 pg/m^3 and 5-189 pg/m^3, respectively (Barber *et al.*, 2007). Additionally, measurements made by the same group of indoor air showed much higher levels of FTOHs, in the area of ng/m^3. Taking into consideration that modern human beings spend most of their time indoor, the exposure through inhalation is most likely much higher than estimated by Martin *et al.* (2005). Food stuff may be contaminated via packaging material or during preparation. However, no data on amounts of FTOH in food could be obtained. Microwave popcorn is wrapped in grease repellent paper containing fluorotelomers, and some of these FTOHs are released during popping of the popcorn, which can lead to inhalation of FTOH and transfer of FTOH to food (Sinclair *et al.*, 2007). This topic is described in more depth below (chapter 1.1.2).

1.1.2 Perfluorinated octanoic acid (PFOA)

Perfluorinated octanoic acid (PFOA) is one of the two PFCs most commonly found in environmental samples and is also one of the most studied PFCs. PFOA is a fully fluorinated compound, i.e. all hydrogen atoms have been replaced by fluor atoms (figure 3).

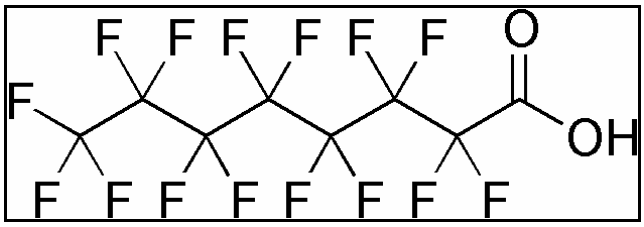

Figure 3: Structure formula of perfluorinated octanoic acid (PFOA)

Use and sources

PFOA is a strong surfactant that is mainly used as a reactive intermediate, while its salts are used as processing aids in the production of fluoroelastomers, fluoropolymers, and other surfactants. PFOA is released directly into the environment, but is also the ultimate degradation product of several precursors such as 8:2 FTOH (Dinglasan *et al.*, 2004). The estimated global production of PFOA in 2003 was 1200 metric tons (Lau *et al.*, 2007). The major PFC-producing companies on a global basis have committed themselves to reducing emission of PFOA and related chemicals by 95% by 2010 in the stewardship program collaboration with the US Environmental Protection Agency (EPA) (Lau *et al.*, 2007). Typical levels in outdoor air are 1-818 pg/m^3 (Barber *et al.*, 2007).

In 2007, the Norwegian Pollution Authority (Statens Forurensninsgstilsynet, SFT) estimated the annual emission of PFOA in Norway, the results are presented in table 1 (SFT, 2007). Their survey concluded that long range transport of PFOA is the most important source of PFOA in Norway. The major direct source from consumer products seems to be impregnated carpets. SFT has suggested to ban the use of PFOA in consumer products in Norway (SFT, 2008a).

The EU has proposed the classification of PFOA as

- toxic (T, R48/23: Toxic: danger of serious damage to health by prolonged exposure through inhalation),
- a reproductive toxicant (Repr Cat 2, R61: May cause harm to the unborn child)
- carcinogenic (Carc Cat 3, R40: Limited evidence of a carcinogenic effect)

- irritant (Xn, R20/22: Harmful by inhalation and if swallowed; R48/22: Harmful: danger of serious damage to health by prolonged exposure if swallowed), and
- irritant to the eye (Xi, R36: Irritating to the eyes) (SFT, 2007; Bjørge, 2008).

Table 1: Estimated annual maximum emission of PFOA from consumer products and long range transport of PFOA in Norway (SFT, 2007)

Carpets	**12 kg**
Coated and impregnated paper	**1.2 kg**
Textiles	**0.5 kg**
Paint and lacquers	**1 kg**
Long range transport	**130 to 380 kg**

PFOA is found as a residual from the production process in polytetra-fluoroethylene (PTFE) films, which are used in non-stick cookware and breathing membranes as in outwear, and as a contaminant in fluorochemical coated food-contact paper as microwave popcorn bags. PFOA has been shown to migrate from food contact paper and non-stick cookware into water and oil (Begley *et al.*, 2005). Begley et al. (2005) found that 4% of the PFOA in the frying pans could migrate into water and oil at 100°C during the first use. The same study measured relatively large amount of PFOA in paper coating and popcorn bags. Sinclair *et al*. (2007) measured PFOA off-gassing from non-stick cookware under normal cooking temperatures (179-233 °C), transfer to water boiled in these pans, and release from microwave popcorn. They found that PFOA is released from cooking pans with a large difference between brands (table 2). The amount released decreased with repeated use in one brands, while it was similar during the first 4 uses in another brand. FTOHs were also released during the first use, but not during repeated use. In addition, PFOA as well as 6:2 FTOH and 8:2 FTOH are released during the popping of microwave popcorn. The amounts released are vary considerable between the different brands (table 3) (Sinclair *et al.*, 2007).

Table 2: PFOA emission during use of non-stick cookware under normal cooking temperature, modified from (Sinclair *et al.*, 2007)

Sample brand	**Gas-phase PFOA released during 1st use (pg/cm^2)**	**Gas-phase PFOA released during 2nd use (pg/cm^2)**	**Gas-phase PFOA released during 3rd use (pg/cm^2)**	**Gas-phase PFOA released during 4th use (pg/cm^2)**	**PFOA retained in water during boiling (ng in 250 ml)**
pan 4	19				<2,5
pan 13	67	27	55	67	<2,5
pan 32	287	145	69	40	75,0
pan 39	61				7,7

Table 3: PFC emission from microwave popcorn, modified from (Sinclair *et al.*, 2007)

Popcorn brand	PFOA measured in the gas phase following popping (ng)	6:2 FTOH measured in the gas phase following popping (ng)	8:2 FTOH measured in the gas phase following popping (ng)	PFOA measured in popcorn paper packaging (ng/cm^2)
PoP 40	16	223	258	6,0
PoP 41	17	< 20	< 20	0,5
Control	<2,5	< 20	< 20	< 0,2

Toxicokinetics

PFOA is readily absorbed after oral intake and inhalation, and can also penetrate the skin (Ophaug and Singer, 1980; Kennedy, 1985). PFOA is mainly distributed to kidney, liver, and blood where it is bound to albumin, but can also be found in the lung, heart, skin, and testis (Vanden Heuvel *et al.*, 1992; Noker and Gorman, 2003). Data by Han *et al.* (2003) suggest that 95% of PFOA in blood is bound to albumin, and Vanden Heuvel and co-workers (1992) suggest that protein sulfhydryl groups are the site of the covalent binding to proteins. PFOA has been reported to bind to β-lipoproteins and liver fatty acid-binding protein, as well as other proteins in liver, plasma and testis (Vanden Heuvel *et al.*, 1992; Luebker *et al.*, 2002). PFOA can cross the placenta (Apelberg *et al.*, 2007b) and enters enterohepatic circulation. PFOA is eliminated mainly in the urine, but biliary excretion and reabsorbtion occurs (Kudo *et al.*, 2001). A study in female rats showed that after a single administration of PFOA, ~90% was excreted in the urine and ~10% in the faeces (Ophaug and Singer, 1980). While urine is the main route of excretion in rodents, faecal excretion predominates in monkeys and humans (Anderson *et al.*, 2008). In rats, PFOA has been found to be transferred to offspring via lactation. The PFOA concentration in the breast milk was approximately 10 times lower than in maternal serum (Hinderliter *et al.*, 2005). Two human studies show some disagreement with respect to transfer of PFOA to human breast milk, with one study showing ppt levels of PFOA (So *et al.*, 2006), and the other finding PFOA levels above the detection limit (0.01 ng/ml, i.e. 0.01 ppb) in only one sample (Karrman *et al.*, 2007).

There is no metabolism of PFOA in organisms (Ophaug and Singer, 1980). The half-life varies significantly in different species, ranging from a few hours in rats and rabbits, to around 20 days in cynomolgus monkeys and several years in humans (Vanden Heuvel *et al.*, 1991; Johnson, 1995; Butenhoff *et al.*, 2004b). The mean half-life in retired exposed workers is about 4 years, with a range of 1.5-13.49 years (Burris *et al.*, 2002). In some animal species large differences in the halv-life of PFOA are observed between the sexes: in female rats the

halv-life is 3.7 hours, while it is 7.4 days in male rats (Kudo *et al.*, 2002; Kemper, 2003). The half-lifes of PFOA in humans and female rats is thus different with a factor of 35,000. The reason for these large species differences are still unknown. In rats, testosterone downregulates the elimination of PFOA (Kudo *et al.*, 2001), while estradiol upregulates the elimination (Ylinen *et al.*, 1989).

Toxicity

The oral LD_{50} of PFOA in rats is 430 mg/kg bw in females and 680 mg/kg bw in males (Griffith and Long, 1980). The difference in LD_{50} value between the sexes is thought to be associated with the shorter half-life of PFOA in female rats.

One of the most distinct effects of subchronic PFOA exposure of murines is an induction of peroxisomal proliferation (PP) through the agonistic effect on PPARα, shown in rats, mice, common carp and zebrafish (Sohlenius *et al.*, 1994; Anderson *et al.*, 2008). The induction of PP is likely responsible for a variety of the effects observed after PFOA exposure since it is believed to increase cell proliferation, possibly inducing cancer development, and increase oxidation of fat (Kudo *et al.*, 2006). After binding of PFOA to PPARα a heterodimers with the retinoid X receptor is formed. This complex is then capable of regulating genes exhibiting a peroxisome proliferator response element (PPRE). Many genes responsible for lipid homoeostasis are regulated by PPREs (Mandard *et al.*, 2004). Exposure to PFOA for 21 days altered gene expression of over 500 genes in the liver of Sprague-Dawley rats. The induced genes were mainly involved in transport and metabolism of fatty acids and lipids, while the downregulated genes were related to transport, inflammation and immune response (Guruge *et al.*, 2006). The authors proposed these effects to be due to PFOA having a very similar structure to endogenous fatty acids and thereby causing interactions with the degradation of unsaturated fatty acids. This theory is supported by findings of Luebker *et al.* (2002) who found PFOA to be able to displace fatty-acid molecules bond to Fabp1, a fatty-acid binding protein (Luebker *et al.*, 2002). The induction of peroxisomal β-oxidation without induction of catalase could lead to oxidative stress from hydrogen peroxide produced from acyl-CoA (Guruge *et al.*, 2006). An effect on gene expression was also seen in CD-1 mice exposed to PFOA *in utero*. Approximately 1500 genes in the fetal liver had altered expression, while about 200 genes were influenced in the fetal lung. Most of the altered genes were associated with lipid homoeostasis (Rosen *et al.*, 2007). A similar result was found by Nilsen *et al.* (2008), who exposed Wistar albino rats for 10 days and found altered expression of 441 genes. Rosen *et al.* (2008) examined the effect of PFOA

on gene expression in PPARα-null mice. A dose of 3 mg/kg significantly altered the expression of 879 genes in wild-type mice, but only 176 genes in the knockout mice. This suggests that the main effect of PFOA on gene expression is mediated by PPARα activation, but that there also are effects on gene expression independent of PPARα. Genes altered in the knockout mice were related to fatty acid metabolism, cell cycle progression, inflammation, and xenobiotic metabolism (Rosen *et al.*, 2008). In a similar experiment, Wolf *et al.* (2008) exposed wild-type and knockout mice to PFOA and measured liver toxicity. Hepatomegaly was observed in both wild-type and knockout mice, while peroxisome proliferation was dependent on PPARα. Hepatocellular proliferation increased in a dose-dependent manner in wild-type mice, while it was only seen at high PFOA doses in knockout mice. The authors hypothesized a possible alternative nuclear receptor-mediated pathway at high PFOA doses leading to a mitogenic response not requiring PPARα receptor (Wolf *et al.*, 2008). Yang *et* al. came to a similar conclusion after observing liver enlargement following PFOA exposure in PPARα-null mice (Yang *et al.*, 2002).

There is though some disagreement concerning the genotoxic effect of PFOA; it has tested negative in the Ames test at concentrations up to 500 μM, suggesting no mutagenic effect (Freire *et al.*, 2008). It has also tested negative in several genotoxicity studies (Litton Bionetics, 1978; Hazleton, 1995; Toxicon, 2002), but Yao *et al.* (2005) showed that PFOA was genotoxic in the human hepatocellular liver carcinoma cell line HepG2 cells, inducing single strand breaks and generating ROS. They hypothezied, however, that the genotoxic effect might be a secondary effect of the ROS generation (Yao and Zhong, 2005).

Although it appears that PFOA is not directly genotoxic, it has been shown to cause an increase in incidence in liver tumours, pancreatic acinar cell tumors, fibroadenomas of the mammary gland, and testicular Leydig cell adenomas in rats at 300 ppm (Riker, 1987; Biegel *et al.*, 2001; Anderson *et al.*, 2008). Both hepatic tumor, Leydig cell-tumor and pancreatic acinar cell-tumor are often observed following exposure to PPs (Reddy and Rao, 1977; Cook *et al.*, 1999). A possible mechanism for the induction of Leydig cell tumor is the alteration of gene expression regulated by PPREs, which are responsible for hormone homeostasis. An induction of CYP19A1 causes an increase in estradiol, which is thought to induce Leydig cell tumor (Cook *et al.*, 1999; Biegel *et al.*, 2001). Another possible explanation for cancer induction in Leydig cells is that the disturbed testosterone biosynthesis after exposure to PPs leads to reduced testosterone levels, which leads to a compensatory increase in lutenizing hormone. This leads then to increases Leydig cell proliferation (Liu *et al.*, 1996; Clegg *et al.*, 1997; Biegel *et al.*, 2001).

Several authors report effects of PFOA on reproduction, including delay in growth, development, eye-opening and onset of puberty in offspring (reviewed by Lau *et al.*, 2004). In addition, *in uterus* exposed mice died soon after delivery (Lau *et al.*, 2006). Some of these effects seem to be PPARα dependent, while other effects such as prenatal lethality were seen in PPARα-null mice as well (Abbott *et al.*, 2007). Decreases in weight and reduced weight gain can be due to the PP effect which leads to increased oxidation of fat.

Whether these toxicity findings in rodents are relevant for human toxicity is yet unclear. PPARα-activation is most likely not a relevant mode-of-action in humans because of the much lower expression in humans compared to rats and major differences in downstream response elements (Cheung *et al.*, 2004). For instance Morimura *et al.* (2006) showed that an induction of the mouse PPARα receptor lead to inhibition of apoptosis, while activation of the human PPARα receptor stimulated apoptosis. Therefore, several of the effects observed in rodents following PFOA exposure might not accure in humans.

Due to the large differences between murines and humans with respect to response to PPARα inducers, PFOA has been tested in *in vivo* experiments using monkeys. Decreased thyroid levels, increased liver weight and hepatic changes, as well as marked diffuse lipid depletion in the adrenals were observed (Griffith and Long, 1980; Butenhoff *et al.*, 2002). PFOA has also been shown to induce apoptosis in the monkey kidney-derived cell line Vero at concentrations of 50 μM for 24 hours (Freire *et al.*, 2008). An increase in intracellular H_2O_2 levels and thereby oxidative stress was also observed.

PFOA seems to alter membrane architecture (Levitt and Liss, 1987) and increase the nonselective permeability of mitochondrial membranes, possibly leading to metabolic wasting (Starkov and Wallace, 2002). The effect on membrane fluidity can possibly be caused by the downregulating influence PFOA has on cholesterol synthesis (Guruge *et al.*, 2006). Another hypothesis is that PFOA acts as a strong detergent and disrupts the inner membrane of the mitochondria, thereby decreasing respiratory control, increasing oxidative stress, and possibly causing apoptosis (Panaretakis *et al.*, 2001). In the human hepatocellular liver carcinoma cell line (HepG2), PFOA induces generation of ROS and mitochondrial hypergeneration at 400 μM for 3 hours (Panaretakis *et al.*, 2001).

PFOA has also been shown to be toxic to aquatic organisms. For instance, exposure to PFOA for 24 hours reduced viability in primary cultured hepatocytes from freshwater *Tilapia* and induced ROS generation, as well as reduced glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activity. After 48 hours, typical signs of apoptosis were observed (Liu *et al.*, 2007b). An induction of vitellogenin production was also observed,

suggesting an estrogenic effect of PFOA (Liu *et al.*, 2007a). This is supported by findings in an *in vivo* study in rare minnow, where PFOA exposure upregulated ER-β (Wei *et al.*, 2007).

Human exposure

Humans are exposed to PFOA via both air, water and food. PFOA has been detected in a variety of food stuff (Smith, 2001; Ericson *et al.*, 2007). The highest estimated exposure occurs in toddlers, who might get doses up to 128 ng/kg/d, while adults are expected to be exposed to 2-3 ng PFOA/kg/d. The higher exposure of children is due to the relatively higher food consumptions and the higher hand-to-mouth transfer of chemicals from treated furniture and carpets and ingestion of dust. Exposure from contaminated food and water is likely the most important sources, whereas house dust and consumer products contributes to a lesser degree. Of the consumer products, impregnation sprays, treated carpets and coated food contact paper are expected to be the major sources (Trudel *et al.*, 2008).

Health Benchmarks for chronic exposures under which no adverse effect are expected are 3.9 mg/kg/day for noncancer systemic toxicity, 22 mg/kg/d for developmental effects, and 5.1 mg/kg/d for carcinogenic effects (Washburn *et al.*, 2005). The estimated intake by Trudel et al. (2008) of less than 150 ng/kg/d is well below these benchmark values. Washburn *et al.* (2005) calculated Margin of exposure (MOE) values based on the benchmark values and estimates of daily exposure through consumer products, and found all MOEs to exceed the range of 100-1000 by at least a factor of 30. They concluded therefore that no adverse health effects are anticipated to be caused by consumer products (Washburn *et al.*, 2005).

PFOA-levels found in serum in the general population are around 5 ppb (Olsen *et al.*, 2003b; Lau *et al.*, 2004; Emmett *et al.*, 2006). Occupational exposure occurs in factories producing PFC-containing products. These workers are often exposed to much higher doses than the general population and the serum levels are one order of magnitude higher (Lau *et al.*, 2007). Temporal trends in the levels of PFOA in human serum are inconclusive, as some studies find an increase (Harada *et al.*, 2007) while others find a decrease (Olsen *et al.*, 2007).

Epidemiology

Studies trying to relate exposure to PFCs levels in humans to different health effects are generally inconclusive or conflicting. In some studies of occupationally exposed individuals a relationship between PFOA concentration in serum and 1) cholesterol and bilirubin levels (Sakr *et al.*, 2007) or 2) cholesterol, triglyceride and tyroid hormone (T3) levels are observed (Olsen *et al.*, 2003a), while other studies find no correlation (Ubel *et al.*, 1980; Gilliland and Mandel, 1996).

A retrospective cohort mortality study found an elevated prostate cancer ratio in production workers, with a 3.3-fold increase in prostate cancer mortality per 10 years of employment (Gilliland and Mandel, 1993). However, another study did not find a correlation between the employment time and PFOA serum concentration (Olsen *et al.*, 2003a). A follow-up study divided workers into categories of likelihood of exposure and found no association between employment time and prostate cancer in the definitely exposed group (Alexander *et al.*, 2003).

A community exposed to higher amounts of PFOA than the general population was surveyed by Emmett *et al.* (2006). The higher exposure was due to a neighbouring industrial facility producing fluoropolymers and resulted in median serum levels of 350 ppb. They found no significant relationship between PFOA serum levels and liver or renal function, cholesterol, tyroid-stimulating hormone, white cell or platelet counts (Emmett *et al.*, 2006). Since the average blood level was 70 times the level found in populations elsewhere, the authors suggest that PFOA does not influence any of the studied parameters in the general population.

To investigate the reproductive effect of PFOA in humans several authors have studied the relationship between maternal PFOA levels and health parameters. Apelberg *et al*. (2007a; , 2007b) found a statistically significant relationship between the level of PFOA in cord blood with birth weight, ponderal index and head circumfence. An increase of PFOA concentration from the 25^{th} to the 75^{th} percentile was associated with a 58 g decrease in birth weight (Apelberg *et al.*, 2007b). In addition, Fei *et al*. (2007) found that birth weight was correlated with PFOA concentration in maternal serum. Infants born to mothers with PFOA levels in the highest quartiles has an average birth weight that was ~100 g lower than infants born to mothers in lowest quartile (Fei *et al.*, 2007).

1.1.3 Summary PFCs

Polyfluorinated compounds are used in a wide variety of consumer products, leading to a wide-spread contamination with these compounds as well as continuous exposure of humans. Fluorotelomer alcohols are volatile compounds, mainly found in the air. Thus, inhalation is the most important exposure route for these compounds. 8:2 FTOH is metabolized rapidly, mainly to O-glucuronides and O-sulphates, and to a lesser part to PFOA. Effects of 8:2 FTOH exposure include hepatocellular necrosis and peroxisomal proliferation, as well as detrimental effects on development in mice. In addition are both 6:2 FTOH and 8:2 FTOH suspected to act as xenoestrogens, so far this has only been shown in *in vitro* studies. PFOA is not

metabolized in the organism and is mainly found bound to proteins throughout the organism. PFOA induces peroxisomal proliferation. As a secondary effect of this, reduced body weight, induction of cancer in the liver, pancreas and testicle are observed. Some of the effects observed after PFOA exposure are though seen in PPARα-knockout mice as well. In addition, PFOA acts as a developmental toxicant. Exposure to PFOA occurs both by food, drinking water, and dust. However, exposure to both FTOHs and PFOA seems to be well below levels at which adverse effects on human health are expected. Nonetheless, due to large species differences in toxikokinetics and mode of action, it cannot be excluded that human health is impaired because of PFC exposure.

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1.2 A literature overview of a brominated compound

1.2.1 1,2-dibromo-3-chloropropane (DBCP)

Use and sources

DBCP is a pesticide used as a soil fumigant against nematodes since 1955. It was sold under the names Fumazone and Nemagon and has been widely used on citrus fruits, grapes, pineapples, peaches, tomatoes, banana palm trees and soybeans (Whorton and Foliart, 1983; ATSDR, 1992). DBCP is applied by metering into the ground or by adding to the irrigation water, it then volatilizes and fumigates the soil. In 1969 over four million kg of DBCP were produced in the USA (Jones *et al.*, 1979), while the annual use worldwide was estimated to 14.7 million kg DBCP prior to the ban in 1979 (ATSDR, 1992). DBCP has a vapour pressure of 0.58mm Hg at 20 °C, a melting point of 6°C and a boiling point of 196°C (reviewed in Rice (1999)) (figure 4).

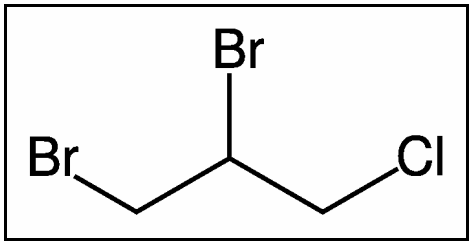


Figure 4: Chemical structure of 1,2-dibromo-3-chloropropane (DBCP)

DBCP is one of the few chemicals that has conclusively been shown to reduce fertility or cause sterility in humans (Whorton *et al.*, 1977; Whorton and Foliart, 1983). The first report of the toxicity of DBCP was published in 1961 (Torkelson *et al.*, 1961), and the following signs of toxicity were reported in rats, rabbits and guinea pigs:

- irritation to the eyes and the respiratory tract
- necrosis of the skin after repeated application
- depression of the central nervous system
- damage to the kidney
- severe atrophy and degeneration of the testis in guinea pigs, rabbits and rats
- reduction in testicular weight
- reduction of spermatogenesis
- development of abnormal sperm

Torkelsen therefore suggested that “a close observation of the health of people exposed to this compound should be maintained” (Torkelson *et al.*, 1961). This advice was obviously not

followed, since the first report of effects in humans were made by the workers themselves after observing that very few of them had fathered a child since they started working with DBCP. A survey of these men revealed azoospermia, oligospermia, raised levels of follicle stimulating hormone (FSH) and reduced levels of lutenizing hormone (LH) (Whorton *et al.*, 1977). Whorton and co-workers concluded that "although the connection has not been proved beyond doubt, the cause in these cases seems to be exposure to the nematocide, 1,2-dibromo-3-chloropropane (DBCP)". DBCP was banned in 1979 in the USA due to sterility observed in production workers. In some developing countries, it has been used until the 1980s (Thrupp, 1991) and it might still be in use in some places today.

Toxicokinetics

DBCP is absorbed after oral, dermal and respiratory administration, and is quite rapidly eliminated with a half-life of 2.37 hours in the rat; the main elimination route of the metabolites is through the urine (Gingell *et al.*, 1987). Låg *et al.* (1989a) found that the concentration of DBCP was higher in the testicle than in plasma.

DBCP can be metabolized via two different pathways: via P450 enzymes or by a glutathione (GSH) S-transferase dependent pathway (Søderlund *et al.*, 1995) (figure 5). In the liver, the cytochrome P-450 dependent pathway leads to the formation of 2-bromoacrolein (2-BA) by oxidation of either C-1 or C-3 (Omichinski *et al.*, 1988b). 2-BA is a potent direct-acting mutagenic compound (Segall *et al.*, 1985). As can be seen in figure 5 both the P450-dependent pathway as well as the GST-pathway can lead to reactive epoxide ions which are electrophilic and can form adducts with macromolecules such as DNA. DBCP has been found to bind covalently to DNA (Omichinski *et al.*, 1988a). Humphreys *et al.* (1991) suggest that the episulfonium ion intermediates form adducts at the N^7-guanine. They also propose that DBCP might be able to induce intrastrand cross-links by reacting with two guanines. However, they report results pointing both towards guanine-guanine links, but also results arguing against the formation of a great number of intrastrand cross-links (Humphreys *et al.*, 1991). The formation of an adduct at the N^7-guanine position leads most likely to an unstable adduct, as described in section 1.3.1.

The GSH-dependent pathway is expected to be more important in testicular cells than the P450-dependent pathway (Søderlund *et al.*, 1988). This theory was supported by the finding that a depletion of cellular GSH blocked the formation of DBCP-metabolites bound to macromolecules as well as the induction of single strand DNA breaks in rat testicular cells (Omichinski *et al.*, 1988a). The levels of GSH S-transferase (GST) activity is higher in rat testicular tissue than in human testis (Dibiasio *et al.*, 1991), suggesting that the GST pathway

is more important in rat testicular cells. The amount of GST has been shown to increase during spermatogenic cell development (Grosshans and Calvin, 1985), this will most likely lead to a higher bioactivation rate in later stages of spermatogenesis and thereby higher levels of DNA damage. Bauche *et al.* (1994) have reported high levels of both GSH and GST in Sertoli cells, while they observed high GSH levels combined with low GST levels in spermatids.

Toxicity

General toxicity

DBCP is acutely toxic and the LD_{50} values for different species are shown in table 4. DBCP acts as a mutagen and clastogen; it induces base-substitutions, but no frameshift mutations (Rosenkranz, 1975). It is a tumor initiator, but not a complete carcinogen for mouse skin (Van Duuren *et al.*, 1979). DBCP has been shown to induce DNA damage in a number of cell types including rat hepatocytes, Chinese hamster cells, rabbit lung cells, human leukaemia cells, pig kidney, rat testicular cells, rat liver cells and human renal proximal tubular cells (Brunborg *et al.*, 1988; Holme *et al.*, 1991; Søderlund *et al.*, 1991; Becher *et al.*, 1993; Brunborg *et al.*, 1996; Wiger *et al.*, 1998). The carcinogenic effect of DBCP is probably caused by its genotoxic effect, most likely by forming adducts with DNA. This theory is supported by positive tests for mutagenicity and findings of DNA damage at the site of the tumour. Several research groups report DNA damage after DBCP exposure. For instance, Brunborg *et al.* (1988) found a dose- and time-dependent induction of DNA damage in rat testicular cells after DBCP exposure. DNA damage is also observed in the foetus of exposed dam (Brunborg *et al.*, 1996). In laboratory animals, an increased incidence rates of cancer in the following organs has been observed after exposure to DBCP: forestomach, mammary glands, nasal cavity, nasal turbinate, tongue, pharynx, adrenal gland, tunica vaginalis, epididymis, cervical node, ovary, skin, lung, spleen, liver, pancreas, kidney (NCI, 1977; NTP, 1982). DBCP is also a potent renal toxicant causing renal necrosis (Kluwe, 1981), has a plasma membrane damaging effect (Omichinski *et al.*, 1987) and inhibits mitochondrial metabolism (Greenwell *et al.*, 1987). DBCP is classified as followes:

- Carcinogen (Car2b, R45: May cause cancer) (IARC, 1999)
- Mutagen (Mut2, R46: May cause heritable genetic damage)
- Reproductive toxic (Repr1, R60: May impair fertility)
- Toxic (T, R25: Toxic if swallowed)
- Irritating (Xn, R20/22: Harmful by inhalation and if swallowed) (SFT, 2008b).

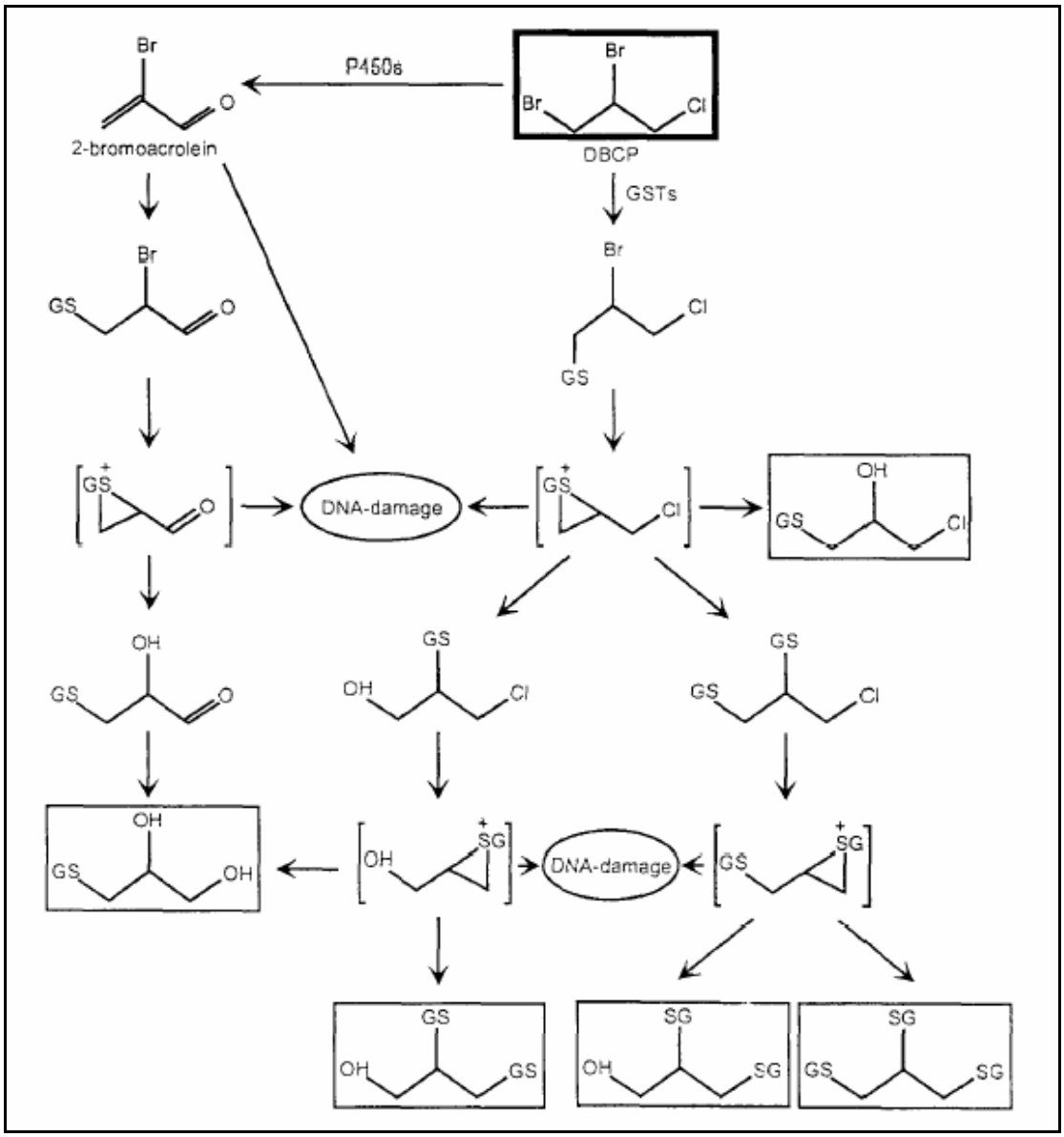


Figure 5: Metabolism of DBCP as proposed by Søderlund *et al.* (1995). Reactive metabolites are shown in open brackets and identified GSH-conjugated in closed brackets.

Table 4: LD_{50} values for DBCP for different species and routes of administration

Administration route	Species	LD_{50}	Reference
Oral	Guinea pig	316 mg/kg	(Whorton and Foliart, 1983)
Oral	Rat	350 mg/kg	(Whorton and Foliart, 1983)
Oral	Rabbit	440 mg/kg	(Whorton and Foliart, 1983)
Oral	Rat	300 mg/kg	(Torkelson *et al.*, 1961)
Oral	Mouse	410 mg(kg	(Torkelson *et al.*, 1961)
Oral	Guinea Pig	210 mg/kg	(Torkelson *et al.*, 1961)
Oral	Rabbit	180 mg/kg	(Torkelson *et al.*, 1961)
Oral	Chicken	60 mg/kg	(Torkelson *et al.*, 1961)
Oral	Prepubertal mice	180.7 mg/kg	(Lee and Suzuki, 1979)
Oral	Adult mice	123 mg/kg	(Lee and Suzuki, 1979)
Dermal	Rat	1.4 g/kg	(Torkelson *et al.*, 1961)
Inhalation	Rat	1 hour: 369 ppm 2 hours: 232 ppm 8 hours: 103 ppm	(Torkelson *et al.*, 1961)

Toxicological effects on the male reproductive system

DBCP reduces fertility in laboratory animals and severely damages testicular cells. A dose of 60 mg/kg led to mild and reversible damage to the seminiferous tubules of rats (Shemi *et al.*, 1982). DBCP reduces lactate production by Sertoli cells and Leydig cell function by acting as an antiandrogen (Miller *et al.*, 1985). No effects were observed in offspring of DBCP treated rats (Warren *et al.*, 1984). The authors suggest therefore that the sperm capable of fertilizing does not contain any DNA damage.

Several research groups report DNA damage in testicular cells after DBCP exposure (Brunborg *et al.*, 1988; Søderlund *et al.*, 1988; Låg *et al.*, 1989b; Bjørge *et al.*, 1996; Labaj *et al.*, 2005). Brunborg *et al.* (1988) report a dose- and time-dependent induction of DNA damage in rat testicular cells after DBCP exposure. Effects after DBCP exposure of testicular cells occur at different doses; reduced mitochondrial function at 250 μM (Greenwell *et al.*, 1987; Bjørge *et al.*, 1995), altered Sertoli function at 300 μM (Bjørge *et al.*, 1995), and DNA damage at 10 μM (Bjørge *et al.*, 1995). Since DNA damage is the outcome detected at the lowest concentration, it is assumed to be the crucial effect.

The effect of DBCP on male fertility has been found to differ between species and between cell types. It is also dependent on the developmental stage of the organism at the time of exposure.

The most sensitive species regarding the detrimental effect of DBCP on male fertility is the rabbit, followed by the rat and the guinea pig (Låg *et al.*, 1989b). The least sensitive are mice and hamster (Foote *et al.*, 1986). A single dose of 40 mg DBCP/kg lead to testicular necrosis and atrophy in rats and guinea pigs, while 120 mg/kg induced only minor damage in mice and none in hamster. The same susceptibility of testicular cells from these species was observed with respect to DNA damage after *in vitro* exposure (Låg *et al.*, 1989b). Låg *et al.* suggest therefore DNA damage to be the initial event in testicular necrosis. When testicular cells from rats were compared to cells from human testis biopsies, short time exposure (30 minutes, 3-300 μM) lead to a distinctive increase in DNA damage only in cells from rats, but not in those from humans (Bjørge *et al.*, 1996). Also, the rate of formation of active metabolites was three-fold higher in rats compared to humans.

Different testicular cells have different susceptibility towards DNA damage induced by DBCP, but there seems to be no consensus on which cell types are the most sensitive. Bjørge *et al.* (1995; , 1997a) found round spermatids to be the most sensitive, followed by spermatocytes and elongated spermatids. Amann and Berndtson (1986) suggest that spermatogonia and spermatocytes are the testicular cell types that are most susceptible to damage caused by DBCP. Lee and Suzuki (1979) found significant unscheduled DNA

synthesis in premiotic germ cells, but not in spermatozoa after exposure to DBCP. However, UDS does not measure induced damage, but repair of the lesions. Meistrich *et al.* (2003) found seminiferous tubules of exposed $LBFN_1$ rats containing no differentiating germ cells, but proliferating and dividing type A spermatogonia. These spermatogonia did not undergo differentiation, but apoptosis, thus suggesting that the oligospermia is not due to a loss of stem cells, but due to a block in differentiation (Meistrich *et al.*, 2003).

Additionally, some stages of development seem to be more susceptible; the effect on the reproductive system was larger when neonatal or adult rats were exposed, than when adolescent rats received the same dose (Kluwe *et al.*, 1985; Saegusa, 1987).

Human exposure

Prior to the ban of DBCP, residues of DBCP were found in several crops. However, the contamination of food today is believed to be minimal, and in 1986 no residues were found in any of over 200 tested foodstuff (Daft, 1989). In areas where DBCP has been used in large amounts like the California's Central Valley and pineapple growing regions in Hawaii, contamination of the groundwater can still be found (Cohen, 1996; Barbash and Resek, 1997). This is due to the long half-life of DBCP in groundwater (about 140 years) (Burlinson *et al.*, 1982). The US EPA has set the Maximum Contaminant Level (MCL) for DBCP in drinking water to 0.2 ppb (0.2µg/l) (EPA, 2002). In addition several states have set guidelines for DBCP content in drinking water, with values reaching from 0.025 ppb (Arizona and Wisconsin) to 0.3 ppb (Minnesota) (Rice, 1999). In surveys done in 1996 in California, DBCP concentrations in drinking water exceeded these guidelines (Cohen, 1996; Kloos, 1996). Wong *et al.* (1988) estimated the dose received via the drinking water in California to be far below the dose associated with infertility in workplace settings and found no signs of reduced fertility in the community studied. Giardino and Andelman (1996) estimated the exposure of DBCP when taking a shower and found that 20% of DBCP in the 40 °C water would vaporize and that the time spent in the shower was proportional to DBCP exposure (Giardino and Andelman, 1996). When DBCP was in use, workers in manufacturing plants, as well as applicators and farmers were heavily exposed, but since DBCP is not in use anymore, occupational exposure is today limited to researchers working with DBCP. Due to preventive measures, as working in closed hoods, this exposure is expected to be negligible.

Epidemiology

As mentioned previously, DBCP is one of the few chemicals that has conclusively been shown to reduce fertility or cause permanent or temporary sterility in humans (Whorton *et al.*,

1977; Whorton and Foliart, 1983). Workers who had been exposed to DBCP showed oligospermia, azoospermia, and elevated levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Whorton *et al.*, 1977). Those who had been exposed for a long time, did not regain fertility even after 8 years (Potashnik and Yanai-Inbar, 1987). However, those who did regain fertility produced healthy offspring. Kharrazi *et al.* (1980) surveyed the pregnancy outcome of wives of DBCP exposed men. They found an increased rate of spontaneous abortion (Kharrazi *et al.*, 1980), while others do not report any increase (Goldsmith *et al.*, 1984). Goldsmith *et al.* (1984) did, however, find a decrease in the male:female ratio in the offspring, suggesting that Y chromosome-bearing sperm cells may be more susceptible to DBCP.

In individuals who had reduced fertility the seminiferous tubules seemed to be the site of damage. An increased proportion of Sertoli cells is observed in individuals exposed to DBCP for longer periods, while seminiferous tubules were devoid of spermatogonia (Biava *et al.*, 1978). This suggests that spermatogonia are the most sensitive cell type to DBCP. In some heavily exposed individuals, Sertoli cells were the only cells found in seminiferous tubules (Biava *et al.*, 1978; Potashnik and Yanai-Inbar, 1987).

Hofmann *et al.* (2006) studied the mortality amongst people who worked on banana plantations in Costa Rica between 1972 and 1979. They found a significant increase in septicaemia and nonsignificant increase in mortality from testicular cancer, penile cancer, Hodgins disease and Parkinson's disease in men. Another research group examined the incidence of cancer in workers from banana plantations in Costa Rica and found an increased standardized incidence rate (SIR) for melanoma and penile cancer (Wesseling *et al.*, 1996).

1.2.2 Summary DBCP

DBCP is absorbed both orally and dermally and metabolized to several reactive compounds. The bioactivation by P450 enzymes is likely most important in the liver, while activation by GST is important in extrahapatic tissue. GSH conjugation leads to the formation of an episulfonium ion, which can attack DNA, causing DNA damage. The most severe effects after DBCP exposure are renal failure and impaired fertility in men, both effects are likely due to the DNA damaging effect of DBCP. DBCP is most likely not in use anymore, but due to the compound being persistent in groundwater deposits, it can still be found in drinking water in some areas. Nonetheless, the exposure of the general population is likely low and not posing harm to human health.

1.3 Testicular cells

The male reproductive system consists of

- the testis
- the external genitalia: penis and scrotum
- the internal genitalia: accessory glands and ducts

The testis is divided into approximately 250 lobule testis by the septula testis. Each lobules contains 1-4 tubuli seminiferi with connective tissue, blood vessels, nerves and intestinal cells as Leydig cells in between (figure 6). Each seminiferous tubule is 30-70 cm in length and contains Sertoli cells and germ cells. The seminiferous tubule merge into tubuli recti (epididymis) and then into the ductulus deferens, also known as vas deferens, from where sperm cells are transported to the urethra (reviewed in Junqueira and Carneirom, 1996).

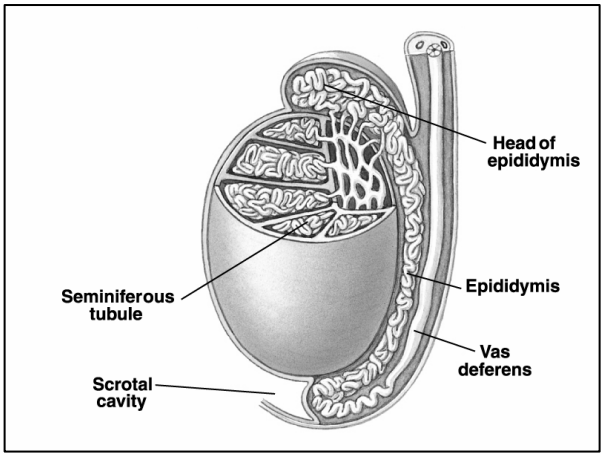


Figure 6: Schematic presentation of the testis (Silverthorn and Ober, 2007)

The **seminiferous tubule** is divided into two parts: the basal compartment and the adluminal compartment. The basal compartment contains the basement membranes, type-A spermatogonia and B-spermatogonia, as well as Sertoli cells. **Sertoli cells** have several tasks: they supply the differentiating germ cells with nutrients, they produce proteins such as enzymes and androgen-binding protein (ABP), they phagocyte remnants from developing spermatocytes, and shield the cells from endogenous and exogenous attack. The protective effect is mediated by the **blood-testis barrier (BTB)**. The BTB consists of a an efflux-pump barrier, an immunological barrier, consisting of Fas ligands on Sertoli cells, and a mechanical barrier, formed by the tight junctions between Sertoli cells and the capillary endothelial cells

(reviewed by Bart *et al.*, 2002). Several transporter proteins have been found be expressed in Sertoli cells and are therefore expected to contribute to the BTB: Mrp8, Tst1, Tst2, Ent1, Ent2, Mrp1, 5 and 7, Mdr2, Oatp3, Oat2, OctN2, Dmt1, Menke`s, Wilson`s, and Znt1 (Wijinholds *et al.*, 1998; Augustine *et al.*, 2005). Some of these are described in more depth in chapter 1.5. During differentiation, primary spermatocytes penetrate the BTB and reach the adluminal compartment, where the later stages of spermatogenesis occur. The more differentiated a cell is, the closer to the luminal end of the Sertoli cell it can be found (figure 7) (Junqueira and Carneirom, 1996). The number of differentiating germ cells is limited by the number of Sertoli cells, with one Sertoli cell being able to support 30-50 germ cells (Weber *et al.*, 1983). Sertoli cells cease dividing when puberty is reached. Sertoli cells contain numerous filaments including vimentin (Franke *et al.*, 1979).

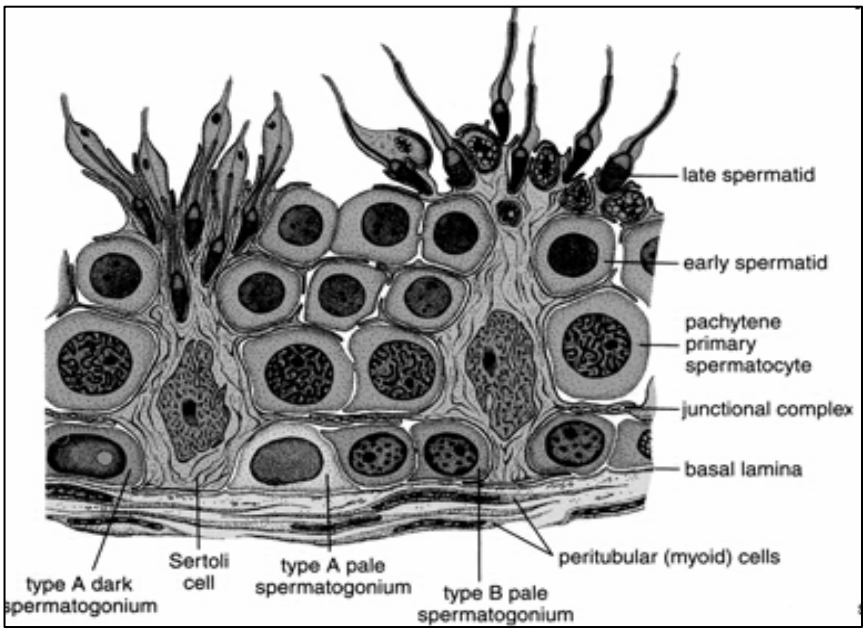


Figure 7: Schematic presentation of cross section of wall of a human seminiferious tubuli (Condon, 2007)

Spermatogenesis is the development of spermatogonia to spermatozoa. Spermatogenesis is a continuous process during the reproductive phase of a male's life. It can be divided into three phases: the spermatogonial phase, in which cells undergo rapid successive division, the meiotic phase in which genetic material is recombined and segregated, and the spermiogenesis phase, in which spermatids transform into spermatozoa (figure 8) (Russel *et al.*, 1990). Prior to puberty, only Sertoli cells, spermatogonia and preleptotene spermatocytes can be found in the seminiferous tubuli. Spermatogenesis takes 74 days in humans and 52 days in rats. It is regulated by FSH, LH and testosterone (Sutovsky and Manandhar, 2006).

In rodents, **spermatogonia** can be divided into spermatogonia A and spermatogonia B. Spermatogonia A can commit to renewal of spermatogonia A by mitotic division, or they

can differentiate into spermatogonia B. Spermatogonia B then undergo spermatogenesis giving rise to spermatocytes, spermatids and eventually spermatozoa. Spermatogonia are found in the basal compartment of the seminiferious tubuli, i.e. outside of the blood-testis barrier (Junqueira and Carneirom, 1996).

Damage to the male reproductive system

An increase in detrimental effects on male reproduction have been observed during the past 60 years in most European countries, but especially in Norway and Denmark (Richiardi *et al.*, 2004). Testicular dysfunction, anomalies and cancer has increased and semen quality has declined. This is probably due to environmental factors and lifestyle factors. The detrimental effect of these factors can be on the germ cells directly, or on the supporting cells (Leydig and Sertoli cells). Factors can influence these cells either directly, for instance by inducing DNA damage, or indirectly, for instance by influencing the hormonal system. The germinal epithelium of the testis is one of the most proliferative active tissues of the body and therefore especially susceptible to DNA damage. DNA damage in germ cells can result in apoptosis of spermatogonia, spermatocytes or spermatids and thereby a reduced number of sperm cells, as well as genetic defects in spermatozoa. Mutations in spermatozoa can lead to pregnancy loss, congenital malformations, mental retardation or childhood cancer, in addition to heritable mutations (Joel, 1966; Cohen, 1986; Colie, 1993; Cordier, 2008) (figure 9). If spermatogonia are damaged fertility is likely reduced permanently, while damage in spermatocytes or spermatids reduces fertility only temporarily. DNA damage in supporting cells like Sertoli and Leydig cells can lead to testicular cancer and a reduction in sperm production.

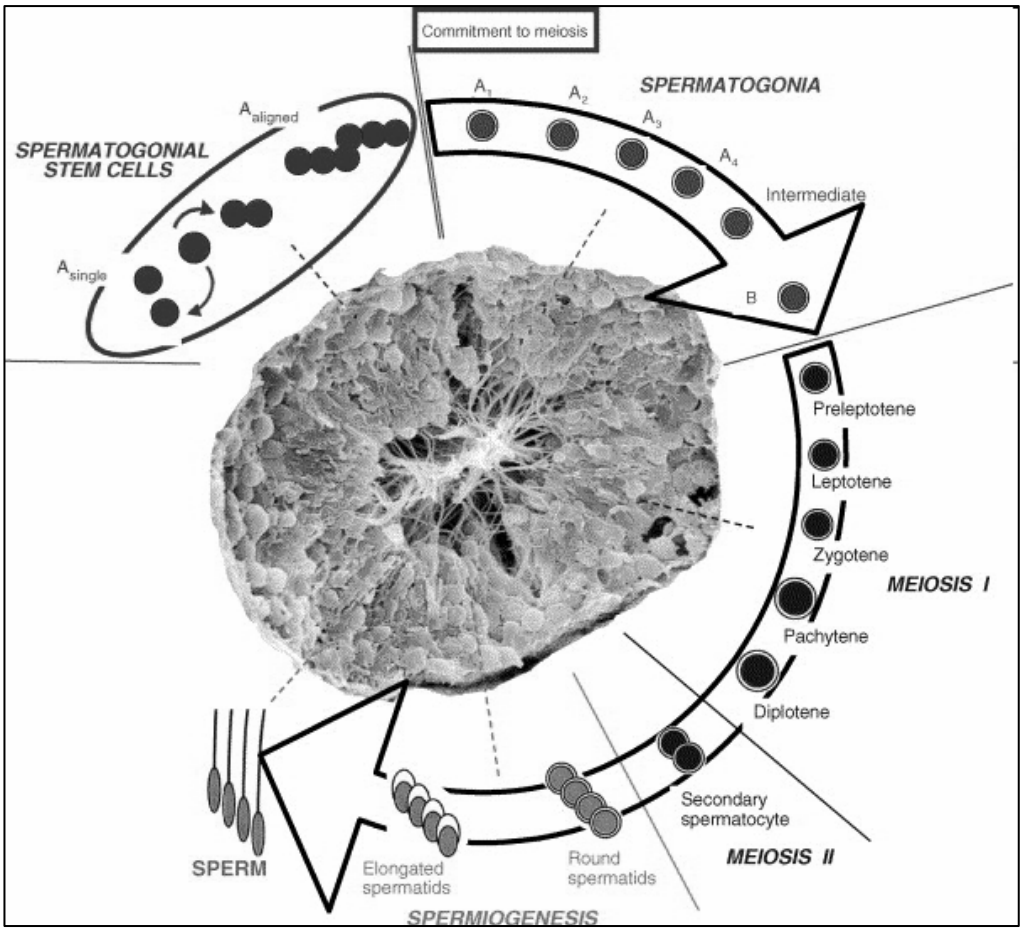


Figure 8: Schematic presentation of spermatogenesis. Spermatogonial stem cell, spermatogonia and later stages of differentiating germ cells are shown. Stippled lines indicate the position of the cell in the seminiferious tubuli (Olive and Cuzin, 2005).

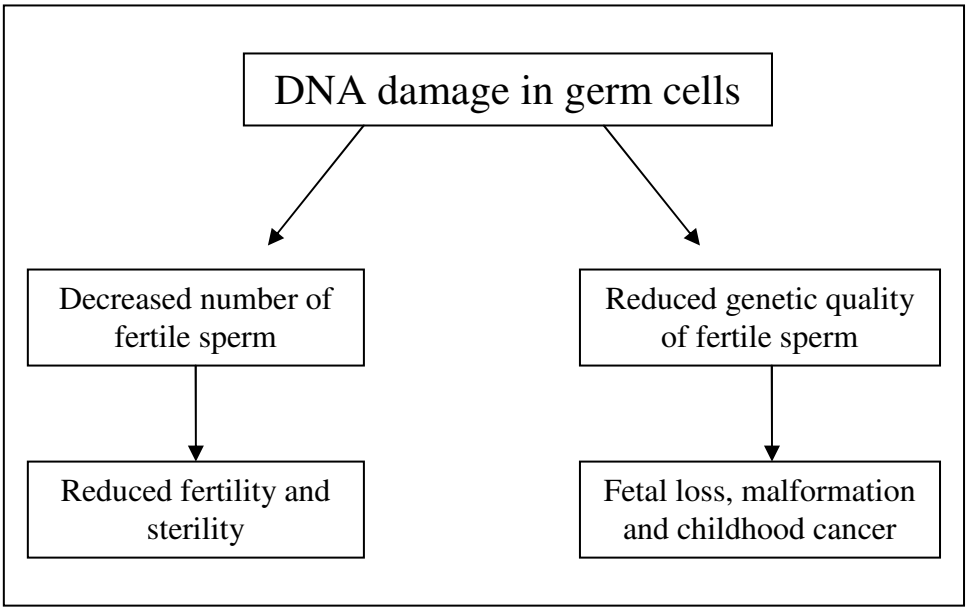


Figure 9: Possible consequences of DNA damage in germ cells (Bjørge, 1996)

1.4 DNA damage

DNA consists of a sugar-phosphate backbone with four different bases attached: guanine, thymine, cytosine and adenine. Three hydrogen bonds are formed between guanine and cytosine, and two hydrogen bonds are formed between adenine and thymine. The DNA holds all the information necessary for the organism to develop and function properly. Damage to the DNA can therefore threaten cellular integrity by affecting vital processes such as transcription and replication, followed by changes in metabolism, cell death, and possibly cancer (Nelson and Cox, 2005).

When the DNA of a cell is damaged, there are basically three possibilities: 1) the damage is noticed by the cell and repaired, 2) the damage is noticed and apoptosis or necrosis is induced, or 3) the damage is not noticed and the cell just "keeps on going". The latter is the most dangerous possibility, since it can lead to alteration of protein function, altered gene expression, or other malfunctions as cancer development. An important distinction is between damage in somatic and germ cells. Many mutations in somatic cells have no effect on the organism as long as they do not lead to the development of cancer, while mutations in germ cells can have severe implications on the progeny (Pierce, 2003). DNA damage includes a variety of lesions spanning from base and sugar damage to DNA-protein cross-links and DNA breaks.

Different mutations have diverse consequences. Point mutations are mutations in one single base. Here we have four different possibilities: transition, transversion, insertion and deletion. Transition is the replacement of a purine with a purine or of a pyrimidine with a pyrimidine. A transversion is the replacement of a purine with a pyrimidine or vice versa. An insertion is the addition of one or more nucleotides, while the remove of one or several nucleotides is called a deletion. Both insertion and deletion can lead to frameshifts if they occur in a reading frame. Frame shift mutations are especially deleterious since they often lead to missense mutations (reviewed in Pierce, 2003).

The phenotypic effects of the change in the DNA can be missense, nonsense, silent and neutral. A missense mutation leads to the incorporation of a different amino acid into the protein. This can lead to a non-functional or a dysfunctional protein, but can possibly lead to a fully functional protein. A nonsense mutation changes a codon for an amino acid into a stop codon, leading to a truncated or maybe non-functional protein produced. A silent mutation is the change of a codon into another codon that codes for the same amino acid. Here the same protein is produced. An example is a change from TCG to AGC. Both code for the amino acid serine. A neutral mutation changes the amino acid sequence in the protein, but does not alter its function (reviewed in Pierce, 2003).

Origins of DNA-damage can either be endogenous or exogenous. Endogenous causes can be leakage of electrons from the electron transport chain, as well as different chemicals that are produced during normal cell metabolism and spontaneous errors made during replication (reviewed in Pierce, 2003). Also, dietary deficiencies can lead to DNA damage. For instance, a deficiency in foliate increases the incorporation of uracil in the DNA (reviewed in Friedberg *et al.*, 2006). Exogenous factors that can induce DNA damage are radiation and chemicals, especially electrophilic molecules and substances producing reactive oxygen species.

1.4.1 Types of damage

Single and double strand break

Radiation, oxidative damage and depurination can lead to single and double strand breaks. Single strand breaks (SSB) are also formed as an intermediate step during repair of DNA damage. Single strand breaks are often repaired correctly, while double strand breaks often are misrepaired and thereby lead to cell death or mutations as sister chromosome exchange (reviewed in Pierce, 2003). Terminally differentiated muscle cells are deprived of replication-associated repair mechanisms, thus increasing the likelihood of persisting SSBs (Narciso *et al.*, 2007).

DNA adducts

Electrophilic agents can form covalent bonds with nucleophilic sites in the DNA. One example is the attack of the episulfonium ion formed during metabolism of DBCP, which forms adducts with guanine (Humphreys *et al.*, 1991). For the most part, adducts are formed with the nucleophilic groups of adenine and guanine (Cavalieri *et al.*, 2000). Each base can form covalent bounds with other molecules at several sites. Depending on at which position on the base the adduct is formed, it can either lead to an unstable or a stable adduct (figure 10). Unstable adducts lead to depurination. Depurination of adenine lead often to A → T mutations, while depurination of guanine leads to G → T mutations (Cavalieri *et al.*, 2000). Stable adducts can lead to mispairing during replication, deletion, frame shift mutations and sister chromatid exchange (Heflich *et al.*, 1986). In addition, stable adducts can lead to arrest of replication and thereby cell death. Besides, adducts can also lead to cross-linkage of DNA to proteins and cross-linking of two bases to each other (Van Beerendonk *et al.*, 1992). Many

different adducts are removed via nucleotide excision repair (NER), as described below, since they are often helix distorting.

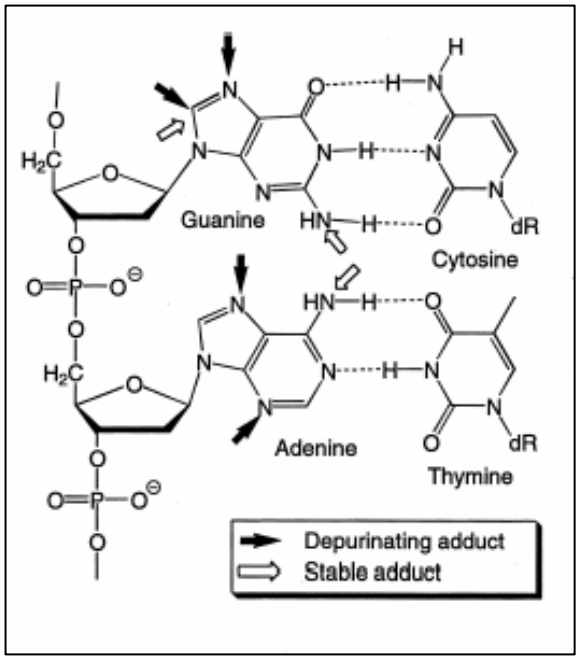


Figure 10: Schematic presentation of a DNA strand with arrows indicating the position of adduct formation. White arrows indicate sites of stable adduct formation, while black arrows indicate sites where unstable adducts are formed, eventually leading to depurination (Cavalieri *et al.*, 2000).

Depurination

Depurination is the loss of a purine (A or G) from the sugar-phosphate backbone of the DNA. This leads to an apurinic site, while loss of pyrimidine (C or T) introduces an apyrimidinic site. The generic term for such DNA lesions is abasic sites (AP site) (figure 11). During replication, a random nucleotide is incorporated opposite to the AP site; usually this is adenine, producing mutations. In addition, the glycosyl bond at an AP site is much more easy hydrolysed, producing a single strand break (reviewed in Friedberg *et al.*, 2006). Depurination can occur spontaneously and as many as 10,000 purines are lost spontaneously from the DNA in a diploid mammalian cell in the course of 24 hours due to hydrolysis of the base-sugar bond (Lindahl, 1979). As described above, depurination can also occur following the formation of an unstable adduct, which, when breaking apart from the DNA, takes the base along. In addition, the formation of AP sites is a reaction intermediates during repair of DNA lesions.

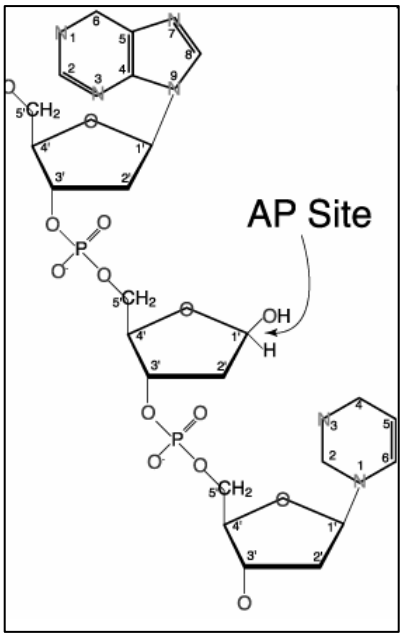


Figure 11: Schematic presentation of an apurinic site (AP) (Huberman, 2006).

Oxidative damage

Oxidative damage is produced by reactive oxygen species (ROS); the most important being hydroxyl radical ($OH^{\bullet}$). ROS are the by-product of normal metabolism, mainly leakage from the electron transport chain (Evans *et al.*, 1997). Other sources of ROS are phagocytosis and peroxisomal metabolism, as well as inflammation and iron overload (Badwey and Karnovsky, 1986; Halliwell and Gutteridge, 2007). In addition, ROS is produced by exogenous factors such as chemicals and radiation. Examples of radiation that produce ROS are UV-radiation, X-ray, and radioactive radiation. A single γ-photon can produce some 36,000 hydroxyl radicals (Breen and Murphy, 1995). Chemicals that can produce ROS include metals, especially iron, that can undergo Fenton reaction and thereby produce $OH^{\bullet}$. Other divalent metals that undergo Fenton reaction include copper and chrome. Oxidative stress has been shown to be one of the major sources of carcinogenesis in epidemiological studies (Toyokuni, 1996; Gilmour *et al.*, 1997; Naito and Yoshikawa, 2005). Defence mechanisms against oxidative damage to cells include antioxidants like superoxide dismutase, glutathione peroxidase, and catalase, several vitamins, as well as repair mechanisms for induced damage.

If an $OH^{\bullet}$-radical is formed in proximity to the DNA it can attack the DNA by 1) addition to the double bonds of DNA bases or 2) oxidation of a base, or 3) abstraction of hydrogen atoms from the deoxyribose sugar units (Cooke *et al.*, 2003). In the latter case, base loss, fragmentation, and strand break can occur. Clusters of hydroxyl radicals can induce double strand breaks (reviewed in Pierce, 2003).

Over 80 products of oxidative damage to DNA bases are known (reviewed in Bjelland and Seeberg, 2003; Cadet *et al.*, 2003; Friedberg *et al.*, 2006), most of which are repaired by

base excision repair (BER) (D`Errico *et al.*, 2008). Guanine has the lowest redox potential among the bases and is therefore most readily oxidized, but attack of the C5=C6 double bond in pyrimidines is also quite frequent. The most studied example due to its biologically importance is the reaction of an $OH^{\bullet}$ with the C8 position of guanine, which leads to the formation of 7,8-dihydro-8-oxoguanine (8-oxoG). 8-oxo-G is strongly mutagenic since it in the *anti* conformation pairs with cytosine, while the *syn* conformation pairs with adenine (figure 12), leading to CG → AT transversion (Shibutani *et al.*, 1991; Kino and Sugiyama, 2005). This lesion is repaired by base excision repair (BER, described below). The first step of the repair of 8-oxoG is the excision of the base by Fpg in bacteria and by the OGG1-DNA-glycosylase in mammals (Barnes and Lindahl, 2004).

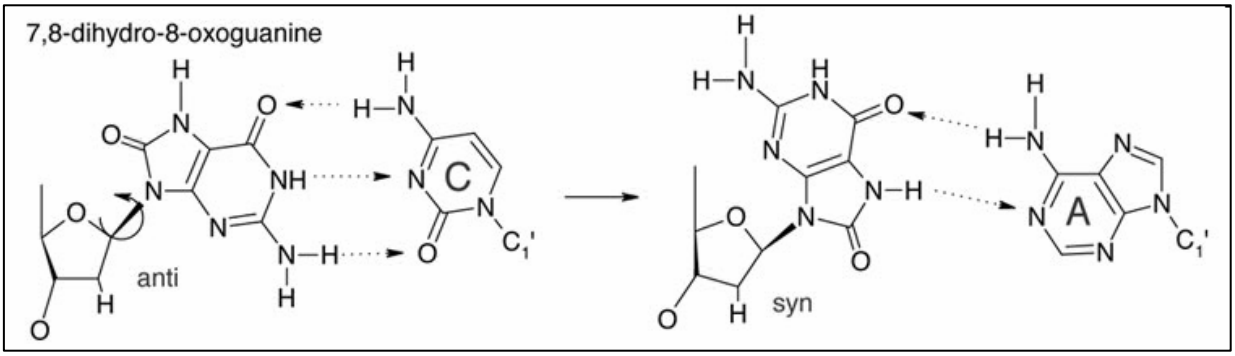


Figure 12: 7,8-dihydro-8-oxoguanine shown in the *anti* conformation, pairing with cytosine, and in its *syn* conformation, pairing with adenine (Horvath, 2002).

1.4.2 Repair of DNA damage

More than 20,000 DNA lesions are estimated to be induced in each diploid cell during 24 hours (Lindahl and Barnes, 2000). The cell has many repair mechanisms to prevent such damage from becoming mutations. The most important repair systems for exogenous induced DNA lesions are base excision repair (BER) and nucleotide excision repair (NER) (Lindahl and Wood, 1999). BER removes small DNA base lesions that do not distort the DNA helix. During BER a glycosylase remove the damaged base by cleaving the N-C1`-glycosylic bond, leaving an abasic site (AP site). An AP endonuclease cuts the phosphodiester bond, DNA ends are trimmed and a DNA polymerase adds a new nucleotide and the nick is sealed by DNA ligase (Krokan *et al.*, 1997). Different DNA glycosylases recognize different DNA lesions; uracil-DNA glycosylase (UNG) cleaves erroneously incorporated uracil, while formamidopyrimidine-DNA-glycolase (Fpg) recognizes ring-opened purines and 8-

oxoguanine, to mention some examples. NER removes helix distorting bulky DNA lesions by separating the strands, cleaving the damaged strand 5 nucleotides upstream and 21-23 nucleotides downstream from the damage. The damaged strand is then removed, the lacking nucleotides are replaced by a DNA polymerase mediated process and the nick is sealed by a DNA ligase.

Repair of DNA damage induced by DBCP in germ cells

Many DNA lesions repaired in somatic cells are repaired poorly or not at all in male germ cells. For instance, Haines *et al*. (2001) detect DNA damage caused by X-ray in epididymal sperm 45 hours after the treatment. The different mechanisms of DNA repair have different activities during the different stages of germ cell development. In murines BER is an active repair mechanism in all stages of germ cell development, but it is likely not an active repair mechanism in human testicular cells (Olsen *et al.*, 2001). A study by Jansen *et al.* (2001) indicated an active nucleotide excision repair (NER) in rat spermatocytes, but no active NER in round spermatids. In contrast to this, Xu *et al.* (2005) studied NER in mice and found a reduction of NER in all postmeiotic cell types, i.e. spermatids and spermatocytes. In humans BER has been reported to be efficient in all germ cells, while NER is not active in human testicular cells (Köberle *et al.*, 1999).

DBCP is metabolized to an episulfonium ion which has been shown to form adducts with macromolecules such as DNA (Bjørge *et al.*, 1996). Humphreys *et al*. (1991) suggest that DBCP forms an adducts at the N^7-position of guanine. Bulky adducts are removed by NER, while smaller adducts also can be removed by BER. Lesions due to DBCP adducts are therefore likely repaired by both BER and NER. In early stages of spermatogenesis, both mechanisms are probably contributing to the repair of DBCP induced DNA damage in rodents. Since NER has been found to be less active in later stages of germ cell development, BER is likely responsible for the removal of DBCP adducts. Bjørge *et al.* (1997b) report that DNA damage induced by DBCP is repaired in spermatocytes, but not in spermatids.

1.4.3 Methods to detect DNA damage and its repair

Several methods can be used to detect DNA damage and its repair, including alkaline elution, unscheduled DNA synthesis, the comet assay (a k a Single cell gel electrophoresis (SCGE)), ELISA, micronucleus, and various chemical methods. The comet assay was chosen in the present study due to the high sensitivity of the assay.

The principle used in the comet assay was first suggested by Ostling and Johanson (1984) and has been modified since to allow for higher sensitivity, detection of specific lesions as

well as higher throughput. The comet assay is used to detect DNA damage and can measure single and double strand breaks, as well as alkalilabile sites such as AP sites. The single strand breaks measured can be due to induced damage, but can also represent intermediate steps during DNA repair. By including repair enzymes as formamidopyrimidine-DNA-glycolase (Fpg), specific DNA lesions can be measured. Fpg recognizes ring-opened purines and 8-oxoguanine and removes the oxidized base, leaving an AP site. The AP site is transformed into a single strand break due to the high pH used during unwinding and thereby measured in the comet assay. Taken together, the alkaline comet assay with Fpg can therefore detect

- single and double strand breaks,
- AP sites, which can be produced by unstable adducts or base loss,
- specific base lesions such as oxidized purines, and
- DNA repair intermediates.

The idea behind the comet assay is that cells are embedded in agarose gel and lysed to remove membranes, proteins and cytoplasm, leaving the DNA as a nucleoid. During electrophoresis, broken DNA, having a negative charge, is pulled towards the anode (positively charged electrode), which leads to a comet-like tail (figure 13). The relative intensity of the tail reflects the frequency of DNA breaks.

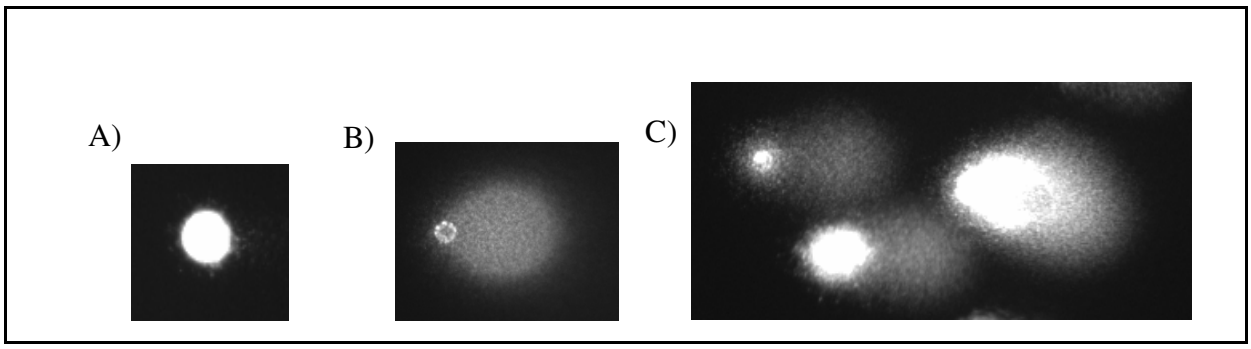


Figure 13: Comet images. Image A) shows a cell with very little DNA damage (approximately 5%tail-DNA); image B) shows a cell with severe DNA damage (approximately 60%tail-DNA). Image C) shows several nuclei that overlap each other. Here the Perceptives software would measure three or four nuclei as one, resulting in an erroneous %tail-DNA. Overlapping nuclei were therefore excluded and not scored.

1.5 Efflux transporters

1.5.1 General background

Absorption, distribution, metabolism, and excretion are highly important in determining the toxicity of a xenobiotic. Cells have developed specialized membrane proteins to be able to protect themselves from harmful substances by transporting unwanted substances out of cells. There are several transporter families, including multi drug resistance (Mdr) proteins, such as P-glycoprotein (P-gp), organic anion transporters (Oat) such as Oat2, metal transporters such as divalent metal transporter, and multi resistance proteins (Mrp). Many of these pumps belong to the adenosine triphosphate (ATP)-dependent cassette (ABC) transporters, which hydrolyse ATP to gain the energy required for the movement of substances across the membrane.

Immaculate function of the effluent pumps is required to maintain low levels of xenobiotics within the cell. Many amphipathic compounds can diffuse into a cell, where they can become conjugated, which makes them more hydrophilic so that they cannot leave the cell by passive diffusion. Without proper function of efflux transporters, these substances would accumulate in the cell (Borst *et al.*, 2000). Inhibition of ABC transporters can lead to increased toxicity of harmful substances, also known as chemosensitization (figure 14) (Epel *et al.*, 2008), while overexpression can lead to resistance (Scotto and Johnson, 2001). Chemosensitization increases the effective dose of drugs and other xenobiotics. Resistance to anticancer drugs has been shown to be related to increased expression of efflux pumps, especially P-gp, which reduce accumulation of the drug in cancer cells (Juliano and Ling, 1976). Efflux pumps are also thought to be important in the clearance of hormone metabolites and other metabolic waste from the cell. Inhibition of the transporters can therefore possibly lead to hormonal disequilibrium. ABC transporters are found in a wide range of tissues important for absorption, metabolism and excretion, as well as at the blood-brain-barrier (BBB) and the blood-testis-barrier (BTB) (Juliano and Ling, 1976).

The expression of selected effluent transporters is studied in this project because inhibition of P-glycoprotein has been shown in mussel cells after exposure to PFOA (Stevenson *et al.*, 2006).

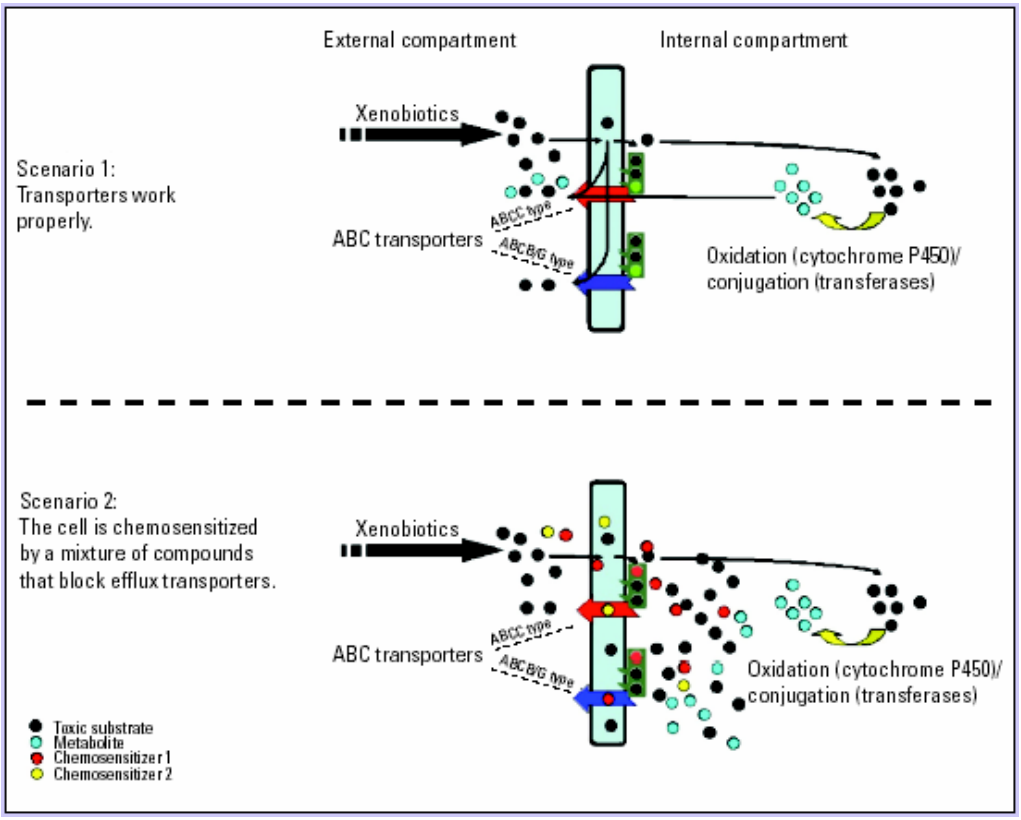


Figure 14: Two scenarios of transporter function in a cell. Scenario 1 shows the normal situation with all transporters working properly. Toxicants are either emitted immediately or after metabolism (oxidation or conjugation). Scenario 2 shows chemosensitization, where the chemosensitizers block the proper function of the transporters, leading to increased amounts of toxic substances inside the cell (Epel *et al.*, 2008).

1.5.1.1 Breast cancer resistance protein (Bcrp1)

The breast cancer resistance protein (Bcrp1, Abcg2) is a membrane protein that belongs to the ABCG-transporter family. It is also known as the Mitoxantrone Resistance Protein (MXR) and as the placenta specific ABC-protein. Bcrp1 is a 655-amino acid-spanning ATP-binding cassette half transporter. It consists of a single hydrophobic membrane spanning domain (MSD) with 6 transmembrane regions, which is preceded by a single nucleotide binding domain (NBD) (Doyle *et al.*, 1998) (figure 15). The NBD binds and hydrolyses ATP to provide energy for the movements of substrates across the membranes (Doyle *et al.*, 1998).

Bcrp1 is involved in the transport of sulphate and glucoronide conjugated organic anions, estrone 3-sulfate and 17β-estradiol 3-sulfate, and phase II metabolites of benzo[a]pyren (Imai *et al.*, 2003; Ebert *et al.*, 2005).

The expression of Bcrp1 seems to be dependent on the aryl hydrocarbonreceptor (AhR) and it is inducible by AhR ligands such as TCDD and PAHs, as well as anticarcinogenic phytochemicals (Ebert *et al.*, 2005; Ebert *et al.*, 2007). Estrogen agonists and antiestrogens were found to have a potent BCRP1 antagonistic activity (Sugimoto *et al.*, 2003).

Bcrp1 is among others expressed in the colon, small intestines, kidney, liver and placenta in humans, as well as in stem cells in mouse testis (Doyle and Ross, 2003; Lassalle *et al.*, 2004; Leslie *et al.*, 2005). Furthermore, Bcrp1 is highly expressed in the mammary gland during pregnancy, possibly playing a role in translocation of essential compounds as riboflavin into the breast milk (van Herwaarden *et al.*, 2007). It is further reported that Bcrp1 limits the penetration of phytoestrogens into the testis, shielding the testis from the estrogenic effect of these compounds (Enokizono *et al.*, 2007). In the human blood-testis barrier Bcrp1 is strongly expressed on the apical side of myoid cells and on the luminal side of the capillary endothelial cells (Bart *et al.*, 2004). In testicular tumour cells, Bcrp1 is more strongly expressed in newly formed vessels than in normal testicular tissue, which can result in resistance towards anticancer drugs (Bart *et al.*, 2004).

The murine form is quite similar to the human BCRP1: they share 81% amino acid identity (Allen and Schinkel, 2002). Human *BCRP1* is mapped to chromosome 4q22 and is regulated by a TATA-less promoter in humans, while it is found on chromosome 6 in mice (Allen *et al.*, 1999; Doyle and Ross, 2003).

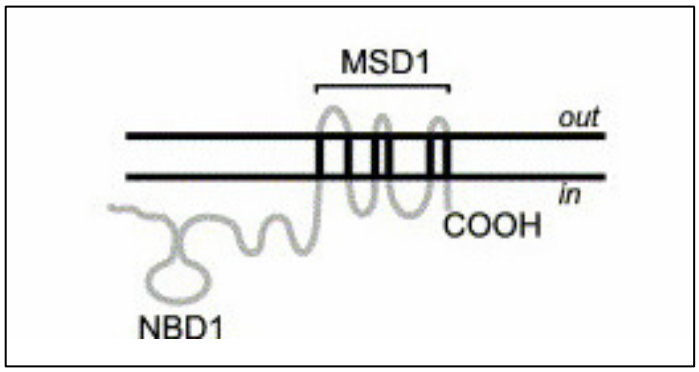


Figure 15: Predicted secondary structure of Bcrp1 (ABCG2). MSD: membrane spanning domain; NBD: nucleotide binding domain (Allen *et al.*, 1999)

1.5.1.2 P-glycoprotein

P-gp (Mdr1, Abcb1) is relatively non-specific with respect to its substrates; it transports mainly lipophilic substrates, but also amphipathic compounds. It is expressed in many tissues, with high levels in the ileum, the blood-brain barrier, the kidney urinary collecting ducts, at the luminal surface of the capillary endothelial cells of testis capillaries, and at somehow lower levels in Sertoli cells (Wijinholds *et al.*, 1998; Choo *et al.*, 2000). P-gp is not expressed in mitotic and meiotic germ cells, but in spermatozoa (Melaine *et al.*, 2002). Inhibition of the P-gp transporter after PFOA exposure has been shown in mussel gill tissue most likely via an effect on the plasma membrane (Stevenson *et al.*, 2006). However, PFOA is not a substrate transported by P-gp, since co-exposure with Verapamil, an inhibitor of P-gp, does not enhance PFOA accumulation in mussel cells (Stevenson *et al.*, 2006). The inhibitory effect of PFOA on P-gp in mussel cells lead to the decision to evaluate the expression of P-gp in testicular cells after PFOA exposure in this thesis.

1.5.1.3 Organic anion transporters

Organic anion transporters (Oats) are expressed in the kidney, liver, brain, and placenta (Sekine *et al.*, 2000). Oats transport organic anions: substances possessing a carbon backbone and a net negative charge at physiological pH. Therefore, it can be assumed that Oats transport PFOA. Oats consist of a 12-transmembrane domain structure (Burckhardt and Wolff, 2000). Oat2 was previously called novel liver transporter (NLT) (Simonson *et al.*, 1994). It is predominantly expressed in the liver, with weak expression in the kidney (Sekine *et al.*, 1998; Sekine *et al.*, 2000). However, the expression of Oat2 in the kidney of female rats has been found to be 7.5 times higher than in male rats (Kudo *et al.*, 2002). Oat2 is expressed mainly in the sinusoidal membrane of hepatocytes, suggesting a role in the uptake of organic anions from the blood into the hepatocytes (Simonson *et al.*, 1994). Oat 2 has also been shown to be expressed in testicular cells and in Sertoli cells (Augustine *et al.*, 2005).

In the rat, PFOA is mainly excreted in the urine, and the excretion is presumed to be mediated by Oats. The renal clearance of PFOA is vastly different in male and female rats, as is the expression of Oats; PFOA is excreted 75 times faster in females, who also show higher mRNA levels of Oat2. Castration of male rats increased PFOA elimination, along with an increase in Oat2 expression (Kudo *et al.*, 2002). An association with Oat3 was also found. Taken together, the results by Kudo *et al.* (2002) suggest that Oat2 and Oat3 are responsible

for the urinary excretion of PFOA. Oat1 and Oat3 have been shown to be inhibited after exposure to PFOA (Hagenbuch, 2008), but the effect on Oat2 has not been examined.

1.5.2 Methods to evaluate gene expression

To evaluate whether the expression of a gene is changed, several methods can be exploited. One can either evaluate gene expression by micro-array, end-point PCR or real-time PCR; or the amount of protein can be measured by immunological methods like Western blotting, or immunostaining and flow cytometry.

Real-time polymerase chain reaction (real-time PCR) was developed by Higuchi *et al.* (1992) and is a sensitive method for the analysis of gene expression. RNA is isolated from cells or tissue and cDNA is synthesised. A master mix, containing a fluorescence marker, for instance SYBR Green, and a heat stable polymerase, as well as primers that are complimentary to the gene sequence of interest, is added to the diluted cDNA. During the real-time PCR this mixture is heated and cooled several times, allowing the primers to anneal and the polymerase to elongate the sequence. SYBR Green binds to the double stranded helix and emits fluorescence which is measured after each round of warming and cooling. During the following heating the two strands partition. Fluorescence is detected during each cycle and, as long as no component has become limiting to the polymerization process, the amount of DNA strands will double each cycle, leading to an exponential increase of the detected fluorescence signal. A threshold value is determined and the number of rounds a sample needs to cross this threshold value is measured (figure 16 A). This value is called the CT (threshold cycle) value for this sample. How quickly a sample reaches this threshold depends on the amount of cDNA for the specific gene sequence in the original sample. It is therefore an estimate of the amount of RNA in the cells or tissue. The CT value is then normalized relative to a housekeeping gene, giving a ΔCT-value. The housekeeping gene is used as an internal control for the PCR. The ΔΔCT-value is calculated by subtracting the ΔCT-value for the exposed sample from the control sample. The $2^{-\Delta\Delta CT}$-value gives a fold-change of the gene expression normalized to the housekeeping gene and the untreated control (Livak and Schmittgen, 2001). This method is based on the following calculations:

The threshold value (CT) is measured during the exponential phase of the real-time PCR. The exponential increase is described by the equation:

$$X_n = X_0 \times (1 + E_x)^n$$

Where X_n is the number of target molecules at cycle number n, X_0 is the number of target molecules at start of the reaction and E_x is the efficiency of target amplification. To calculate the number of target molecules at CT the equation becomes

$$X_T = X_0 \times (1.E_X)^{C_{TX}} \quad \text{for the target gene and}$$

$$R_T = R_0 \times (1+E_R)^{C_{TR}} \quad \text{for the reference (housekeeping) gene}$$

where X_T and R_T are the threshold number of target and reference molecules, C_{TX} and C_{TR} the threshold cycle for target and reference amplification and E_X and E_R the amplification efficiencies, respectively. Dividing these two equations gives

$$\frac{X_T}{R_T} = \frac{X_0 \times (1+E_X)^{C_{TX}}}{R_0 \times (1+E_R) C^{TR}}$$

Assuming equal amplification efficiencies in both reactions (E_X=E_R=E), this equation simplifies to

$$\frac{X_T}{R_T} = \frac{X_0}{R_0} \times (1+E)^{C_{TX}-C_{TR}} = X_N \times (1+E)^{\Delta CT} = K$$

where $X_N = X_0/R_0$, i.e. the amount of the target gene normalized to the reference gene and $\Delta CT = C_{TX} - C_{TR}$, i.e. the differences in CT-values for the target and the housekeeping gene, and K a constant. Rearrangement of this equation gives

$$X_N = K \times (1+E)^{-\Delta CT}$$

The relative X_N of one sample to another is then

$$\frac{X_{N1}}{X_{N2}} = \frac{K \times (1+E)^{-\Delta CT1}}{K \times (1+E)^{-\Delta CT2}} = (1+E)^{-\Delta\Delta CT}$$

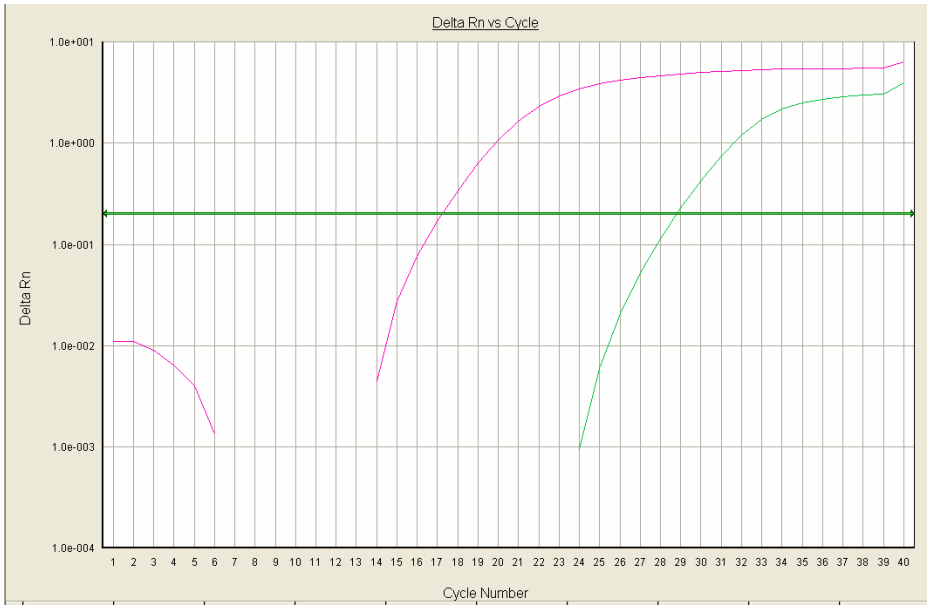
where $\Delta\Delta CT = \Delta CT_1 - \Delta CT_2$. Assuming an efficiency close to one (E=1) this simplifies to an expression for the amount of the target gene in one sample relative to the amount in another sample

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

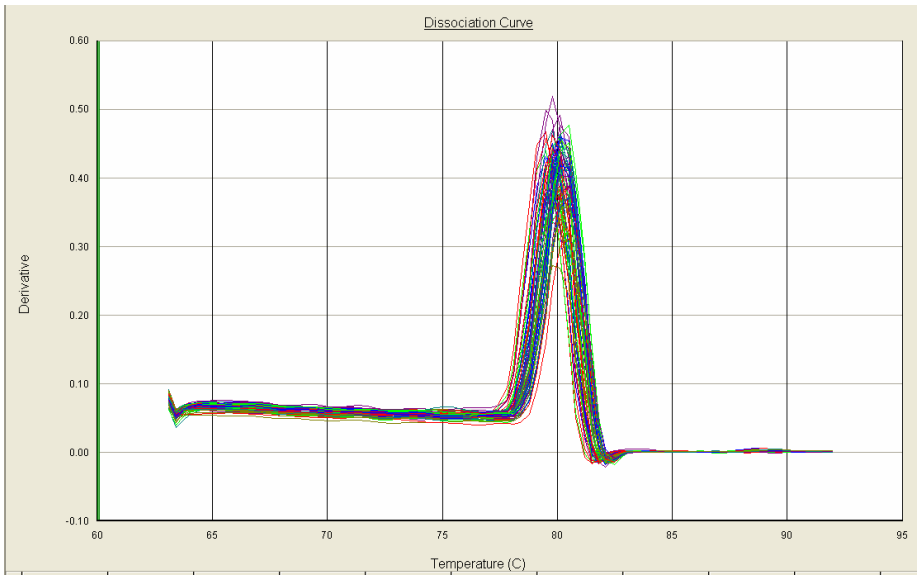
This method was developed by Livak and Schmittgen (2001) and is often referred to as the $2^{-\Delta\Delta CT}$-method.

To control for primer-dimer formation, mispairing, and contamination, a melting curve analysis should be done after the completion of the PCR. Here, the temperature is increased and the fluorescence is measured as a function of temperature. As the temperature rises, hydrogenbonds between the double-stranded DNA molecules get disrupted. The larger the molecule is, the higher the melting temperature will be. At the temperature where the double-stranded DNA separates, the fluorescence signal drops abruptly. This drop of the fluorescence signal is transformed to a peak in the graphical presentation of the derivate of the signal (figure 16 B). Primer-dimer products have lower melting temperatures, making them easily distinguishable from the desired PCR-product. Several peaks in the dissociating curve indicate several products, i.e. unspecific product formation (figure 16 C).

A)



B)



C)

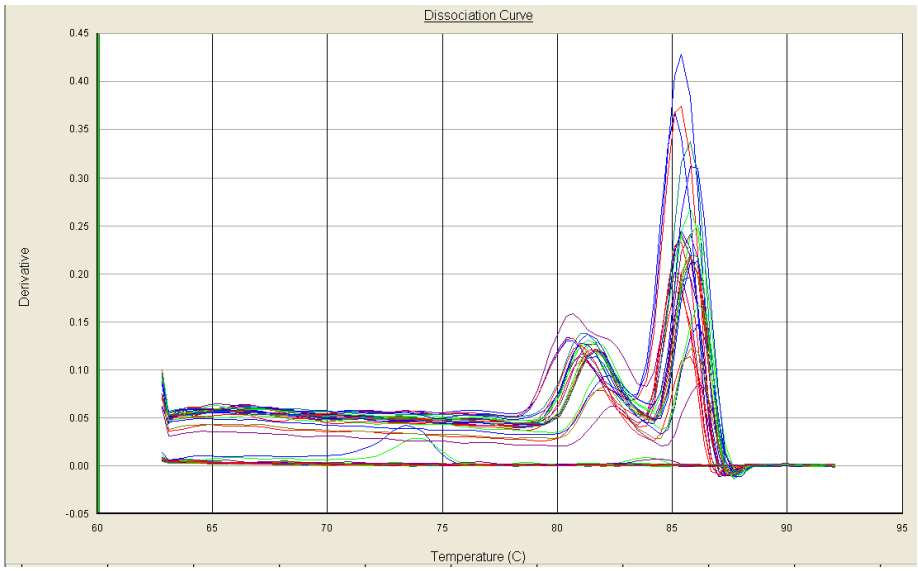


Figure 16: A) Output of a real-time PCR-run. The green line represents the threshold line. The pink line represents a sample with a lower CT-value than the green line. This indicates that there was more RNA of the gene examined in the sample represented by the pink line. B) Dissociation output showing a single product present in all wells. C) Dissociation output with two pikes, indicating several products, and some lines without peaks from wells were no product was formed.

1.6 Hypothesis to be tested

Based on the literature study several hypotheses emerged that should be investigated in the practical part of this study:

1. The polyfluorinated compounds are not expected to induce cytotoxicity or DNA damage after one hour of exposure. This hypothesis is based on findings by Martin *et al*. (2005), Stankowski *et al*. (2001) and Freire *et al*. (2008).
2. Based on findings by Stevenson *et* al. (2006) the polyfluorinated compounds, especially PFOA, are expected to inhibit P-gp and possibly also other effluent transporters. This is expected to lead to increased gene expression of the transporters.
3. DBCP is expected to induce DNA lesions in a dose-dependent manner in all testicular cells, as reported by Brunborg et al. (1988). Based on observations by Bjørge *et al.* (1995) the damage is expected to be less in Sertoli cells than in spermatids. Due to higher GST levels in later stages of spermatogenesis (Grosshans and Calvin, 1985) a higher bioactivation rate and therefore more DNA damage is expected in spermatids than spermatogonia. Thus, the following sensitivity ranking is expected: spermatids > spermatogonia and spermatids > Sertoli cells.
4. The DNA lesions caused by DBCP are expected to be repaired in both spermatogonia and spermatids (Bjørge *et al.*, 1997a). The repair is likely faster in spermatogonia compared to spermatids because of the reduced activity of repair systems in later stages of germ cell development (Xu *et al.*, 2005; Matulis and Handel, 2006).

2. Materials and methods

For a detailed list of chemicals, media and solutions see appendix 7.1 and 7.2. For a detailed overview of experimental design, refer to appendix 7.3.

2.1 Chemicals

1H,1H,2H,2H-Perfluoro-1-octanol (6:2 FTOH, 97% purity, USA) and 1H,1H,2H,2H-perfluoro-1-decanol (8:2 FTOH, 97% purity, Japan) were purchased from Sigma-Aldrich. Perfluoro-n-octanoic acid (PFOA, >98% purity) was purchased from Wellington Laboratories, Ontario, Canada. DBCP (>99% purity) was kindly supplied by Dr. Sidney D. Nelson, University of Washington, Seattle, USA. Refer to appendix 7.4 for CAS number, chemical structure, and chemical name of the compounds.

2.2 Animals

Wistar rats (HanTac:WH) were purchased from Taconic (Denmark) and bred at the facility at the Norwegian Institute of Public Health (Norway) with 12 hour light/dark cycle, 6-10 air changes per hour, controlled humidity (55 ±5%) and temperature (19-23°C). The animals were given Harlan Teklad 2018E diet (Harlan, UK) and water *ad libitum*. The breeding cages contained one male together with two females. Pups were separated at the age of three weeks and only males were used in this study.

2.3 Preparation of cell samples

2.3.1 Preparation of testicular cells

Testicular cells were prepared by the digestive enzymatic method, modified after Søderlund *et al.* (1988). Wistar rats of 5-8 days of age (for isolation of spermatogonia) or 6-8 weeks of age (for isolation of adult testicular cells) were sacrificed by CO_2 asphyxia and the testicles were removed and placed in testicular medium added 10% FCS (see appendix 7.1 for details on media composition). The capsule was removed from the testicles and the tubuli were cut in smaller pieces and incubated in a solution of 100U/ml collagenase in testicular medium for 20 minutes in a rotating water bath at 32 °C and 104 rpm. This step dissolves extratubular cells such as Leydig cells, connective tissue and blood cells. The tubuli were then allowed to sediment and washed twice with medium to remove the dissolved cellular fragments, as well

as blood. The remaining cells were incubated with 100U/ml collagenase once more in a water bath for 20 minutes at 32 °C and 104 rpm. Trypsin was then added to an activity of 2324 U/ml and this solution was incubated for another 8 minutes. This step eliminates the cell-cell-contact, i.e. germ cells are detached from Sertoli cells, and Sertoli cells are detached from each other and the basal membrane. After the incubation, medium containing 10% FCS was added to inactivate the trypsin. After spinning at 1500 rpm for 5 minutes, the cells were resuspended in new medium containing 10% FCS. This washing step was repeated three times to deactivate the trypsin. The cells were then filtered through gaze to remove tubuli fragments, sperm cells, and cell debris, and then through a 100 μM and a 40 μM net. A small sample was stained using Trypan Blue to evaluate viability. Cells were then allowed to recover from the isolation process for half an hour at 32°C, 5% CO_2 before exposure. The cell suspension from adult rats, contain several different testicular cells: primary and secondary spermatocytes, spermatids, Sertoli cells, and Leydig cells. The cells obtained from prepubertal rats consist of Sertoli cells, other somatic cells, and germ cells, of which type A spermatogonia are the most abundant cell type (Clermont and Perey, 1957).

2.3.2 Purification of spermatogonia and somatic cells

Cells from prepubertal rats (5-7 days of age) were isolated from testicular tissue as described above. Purification of spermatogonia was done as described by Morena *et al.* (1996): Cells were sown in plastic dishes coated with lectin and incubated at 32°C for one hour to remove the adhering somatic cells. During this incubation, somatic cells get attached to the lectin, increasing the percentage of spermatogonia in suspension. Different incubation times were investigated, and one hour was found to result in the highest percentage of spermatogonia. After one hour incubation, the suspension was removed from the lectin dishes and placed in bacterial dishes for exposure. An incubation time of 24 hours was found to lead to the lowest percentage of spermatogonia in suspension and was used to examine effects on Sertoli cells and other somatic cells.

The coated plastic dishes were prepared by adding a 5 μg/ml solution of lectin in phosphate buffered saline (PBS), which was incubated for 1 hour at 37°C and washed off with 0,5% BSA. The dishes were then dried and stored at room temperature before usage (Morena *et al.*, 1996).

2.4 Vimentin staining for assessment of cell identity

The amount of somatic cells was evaluated by staining of cells with vimentin, a cytoskeletal protein expressed solely in somatic cells. Spermatogonia were isolated from rat testicular cells as described previously. After spinning at 1500 rpm for 5 minutes, the cells were resuspended in PBS containing 2% PFA at a concentration of 1 million cells/ml. A small drop of this cell suspension (3-5 µl) was applied on a Polysine Microslide and allowed to dry. After washing in distilled water, the cells were incubated over night at 4°C with the primary antibody solution (anti-vimentin clone V9, Dako AS, Denmark) at a 1:2000 dilution in 1% BSA solution with 0,01% thimerosal. The next day, the cells were washed twice in PBS and dried. This was followed by incubation for 30 minutes with the secondary antibody (RRX anti-mouse, Jackson Immuno Research Laboratories, PA, USA) at a dilution of 1:100 in 1% BSA solution with 0,01% thimerosal. The slides were then washed 3 times with PBS. Hoechst 33258 was added to the last wash to stain the DNA. After an additional wash in water, the slides were dried and analysed with Olympus BX51 microscope with an Osram Mercury Short ARC HBO® 100W/2 light bulb. Cells stained with Hoechst and those stained with vimentin were counted manually and the percentage vimentin-negative cells was calculated. The somatic cells, such as Sertoli cells, stain positively for vimentin, so the vimentin negative cells are evaluated to be germ cells. No distinction could be made between spermatogonia A and spermatogonia B.

2.5 In vitro *exposure of cells*

2.5.1 Polyfluorinated compounds and DBCP

Stock solutions of the test compounds were prepared in DMSO and stored at 4°C. Cells were exposed in suspension at 32 °C, 5% CO_2, 20%O_2 for one hour at a density of 1-2 million cells/ml in bacterial dishes. Testicular cells from adult rats were exposed to 0, 3, 10 and 30 µM DBCP, while spermatogonia and somatic cells from prepubertal rats were exposed to 0, 10, 30 and 100 µM DBCP. Exposure with the different PFCs was done with concentrations of 0, 10, 30, 100, 200 and 300 µM. 30 µM DBCP was used as a positive control in the comet assay with polyfluorinated substances. The PFC doses were chosen according to results by Martin *et al.* (2005) using up to 200 µM. Control cells were exposed to 0.2% DMSO. The amount of DMSO was not adjusted in the other treatments. After exposure, cells were put on ice and washed three times with cold PBS to remove excess exposure and to inhibit repair of DNA damage.

2.5.2 X-ray experiment

To standardize the Comet assay, experiments were performed in which cells in suspension were exposed to 0, 3, 6 or 10 Gy of X-ray. The cells were irradiated in eppendorf tubes placed directly on the X-ray tube window, with a 0.5 mm Cu film as filter. The X-ray source was a Phillips MG300 X-ray unit (Germany) operated at 10 mA and 260 kV. The dose rate was 10 Gy/minute, as measured by Fricke's chemical dosimetry. It is expected that 1Gy of X-irradiation causes 0.31 breaks per 10^9 Da of cellular DNA, which is approximately 1000 breaks per diploid mammalian cell (Ahnstroem and Erixon, 1981). The %tail-DNA increases linearly with break frequency over most of its range and %tail-DNA can therefore be converted to number of breaks/cell.

2.5.3 Determination of repair capacity

For the determination of the repair capacity, cells in suspension were exposed to DBCP at 32°C, 5% CO_2, 5% O_2 for one hour and then extensively washed with medium containing 10% FCS. Each cell sample was then divided into 6 subsamples and these were placed in bacterial dishes and incubated at 32°C, 5% CO_2, 5% O_2 for 0, 1, 2, 4, 8 or 24 hours. The DNA damage after the repair was then analysed by the Comet assay by assessing the %tail-DNA (described below).

2.6 Cytotoxicity by Trypan Blue exclusion test

After exposure and extensive washing, cells were stained with Trypan Blue and viability was determined by manual counting in a Buerker counting chamber. Live cells with intact membranes will exclude the Trypan Blue dye, while dead cells do not exclude the dye, and appear blue in the microscope (figure 17). The number of blue cells gives an indication of dead cells and thereby an indication of cytotoxicity. Approximately 80 cells in 5 different squares were counted from each sample in three separate experiments.

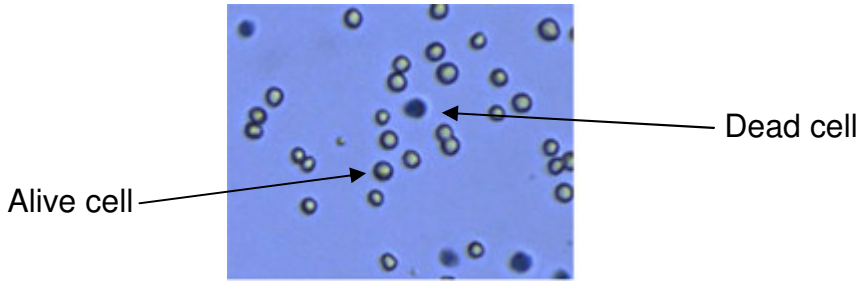


Figure 17: Image showing living cells (white) and dead cells (blue) after staining with Trypan Blue (Qiu, 2008)

2.7 The comet assay

All solutions used in the comet assay are described in appendix 7.2. After the previously described exposure and wash, cells were resuspended in medium at a concentration of 2 million cells per ml. This suspension was then mixed 1:10 with 0.75% low melting agarose at 37°C, which was dissolved in 10mM EDTA in PBS and immediately moulded in wells on a cold GelBond film. Three wells were moulded from each sample, producing 3 gels per sample (refer to appendix 7.3 for overview of experimental design). These gels were then placed in 4 °C lysis buffer over night. This step lyses the cells, most protein is washed out, and only nucleoides remain in the agarose gel. After rinsing in distilled water, the films were transferred to Fpg-enzyme reaction buffer and incubated for one hour at 4 °C. The buffer was changed after the first 10 minutes. After the incubation in cold Fpg-enzyme reaction buffer, half of the films were incubated in Fpg-enzyme reaction buffer with 0,2 mg/ml BSA added 1 µg/ml Fpg crude enzyme, while the other half was incubated in Fpg-enzyme reaction buffer with BSA but without Fpg enzyme, all at 37 °C for one hour. The Fpg enzyme detects oxidative damage by transforming oxidized purines to AP sites, as well as by making an incision next to it. To stop the enzyme reaction and to unwind the DNA, the films were incubated for 5 + 35 minutes in electrophoresis buffer. The supercoiling of the DNA stand is relaxed during unwinding since the high pH disrupts hydrogen bonds between the double-stranded DNA. In addition, the high pH of the buffer (13.2) leads to the transformation of alkalilabile sites, as AP sites, to single strand breaks. Electrophoresis was then performed in the same buffer at 8 °C with 20 V and 300 mA corresponding to 0,74 V/cm for 20 minutes. During electrophoresis, DNA loops are pulled out of the nucleoide. The more strand breaks exist in the DNA, the more loops can be pulled out of the nucleoide. To stop further unwinding, the films were placed in neutralizing solution. After fixating the gels with alcohol, they were dried and stored until scoring. For scoring, the DNA was stained with SYBRGold in TE-buffer for 20 minutes. The comets were scored using either a Leica DMLB with an Osram Mercury Short ARC HBO® 50W/2 light bulb, or an Olympus BX51 microscope with an Osram Mercury Short ARC HBO® 100W/2 light bulb. The Comet assay IV, Perceptives instruments software was used. The %tail-DNA was used as a measure of damage. It is a measure of the relative fluorescence intensity in the head and the tail, and is the suggested measure of DNA damage in the comet assay by the International Workshop on Genotoxicity Test Procedures in 2005 (Burlinson *et al.*, 2006). The %tail-DNA increases linearly with break frequency, is independent of the threshold setting in the image analysis program, and allows measurement of damage across the widest possible range of damage (0 to 100%) (Collins, 2004). In each gel 50 nuclei were scored, since Smith *et al.* (2008) have shown that

an increase to 100 nuclei per gel resulted in virtually no increase in observed power of the statistical analysis (Smith *et al.*, 2008). Overlapping cells were excluded from the analysis, since the software cannot discriminate between overlapping nuclei, leading to an incorrect %tailDNA. This could possibly lead to a biased sampling technique, since comets with small tails are less likely to overlap. However, by closely controlling the cell density in the agarose gels, the number of overlapping cells was minimized.

Cellular samples obtained from adult testicles were found to contain mainly haploid cells, i.e. round, elongating and elongated spermatids. Only round spermatids having a round shaped nucleus with histone-based chromatin were analyzed in the comet assay. Both elongating and elongated spermatids by various degrees of sickle-shaped nuclei were omitted from comet analysis. Subsequently, in this report the term spermatid refers to round spermatids.

2.8 Gene expression analysis

2.8.1 Preparation of cells

Cells are isolated from rat testicles and exposed to PFCs as previously described. After exposure, cells were washed with cold PBS. After the last centrifugation, the PBS is aspirated and about 4 million cells per exposure are stored at -80 °C until isolation of RNA.

2.8.2 Isolation of RNA

Total RNA was isolated using the SV Total RNA Isolation System kit (Promega, USA). The frozen cell samples were suspended in 175 µl RNA lysis solution by pipetting with a syringe. After adding 350 µl RNA dilution buffer, the samples were placed in a heating block at 70 °C for 3 minutes. Spinning at 12000x g for 10 minutes at room temperature leads to precipitation of proteins and salts. The supernatant was then transferred to a new tube and mixed with 200 µl alcohol. This solution was transferred to a spin column assembly and centrifuged at 12000x g for one minute. The RNA on the filter in the spin column assembly was washed by adding 600 µl RNA wash solution and spun for one minute at 12000x g. To remove DNA from the samples, DNase incubation mix, consisting of 5 µl DNase, 40 µl Yellow Core and 5 µl 0,09 M MnCl, was incubated with the RNA for 15 minutes. The process was stopped by adding 200 µl DNase Stop Solution and centrifuging at 12000x g for one minute. DNA and DNase was removed by washing twice with RNA Wash Solution. The Spin basket with the RNA was transferred to a new tube and 100 µl nuclease-free water was added on top. After spinning for

one minute at 12000 g, the solution was transferred to the membrane again, and the spinning is repeated to elute all the RNA. The RNA content in the solution was measured using the Thermo Scientific NanoDrop™ 1000 spectrophotometer, the RNA was aliquoted and the samples were stored at – 80 °C.

2.8.3 Preparation of cDNA

cDNA was synthesised by Reverse Transcriptase (RT) using the Reverse Transcription System kit (Promega, USA). The RNA sample (10µl) was mixed with 18 µl Reverse transcriptase buffer, 36 µl 25mM $MgCl_2$, 18 µl 10mM dNTP, 4,5 µl Rnasin, 9 µl random primers and 6,75 µl AMV enzyme. cDNA synthesis was done in the eppendorf Mastercycler Gradient. The content of cDNA in the sample was measured by the Thermo Scientific NanoDrop™ 1000 spectrophotometer, the cDNA was aliquoted and stored at -20°C.

2.8.4 Quantitative real-time polymerase chain reaction (real-time PCR)

The real-time PCR was used to investigate whether selected genes were up- or downreglated in the exposed cells compared to control cells. The effect on three genes was analyzed:

- Bcrp1
    - forward: 5`-CAATGGGATCATGAAACCTG-3`;
    - reverse 5`-GAGGCTGGTGAATGGAGAA-3` from Kameyama *et al.* (2008),
- Pgp/Mdr1b
    - forward: 5`-ACAGAAACAGAGGATCGC-3`;
    - reverse: 5`-AGAGGCACCAGTGTCACT-3`, 352 bp, from Kameyama *et al.* (2008), and
- Oat2 (Slc22a7, SuperArray).

A dilution experiment was performed to find the best dilution of the cDNA and to evaluate if the underlying assumptions as equal amplification efficiency were complied. In this dilution experiment the cDNA was added to the wells in 5 replicated for each dilution (1, 0.1, 0.01 and 0.001). During subsequent experiments, 2 µl of the diluted cDNA were added in each well of a 96 well plate. The first experiment was carried out using a 1:100 dilution, while the second experiment was done with a 1:10 dilution. Each sample was analysed in 5 replicates for the selected gene and in 3 replicates for the housekeeping gene 18S. Specific master mix was prepared for each gene containing 10 µl Power Sybr Green mix (Applied Biosystem, UK), 6 µl nuclease free water, 1 µl forward primer and 1 µl reverse primer; and added to each well.

The real-time PCR was performed on an Applied Biosystem 7500 Fast Real-Time PCR System with the following configuration:

- Stage 1: 94 °C for 10 minutes.
- Stage 2: 94 °C for 15 seconds, followed by 60°C for one minute and 72 °C for 30 seconds. This stage was repeated for 40 cycles and the fluorescence was detected during the 72 °C-step.
- Stage 3: 95°C for 15 seconds, 60 °C for one minute and 95°C for 15 seconds.

Afterwards, a dissociation stage was run with a gradual temperature increase.

2.9 Statistical analysis

Statistical analysis of cytotoxicity and DNA damage were done with SPSS v16.0. Data from real-time PCR were analysed in Microsoft Excel 2003 and Matlab v7.0, as well as SPSSv16.0. Where ANOVA was used, the three underlying assumptions had to be evaluated:

- Mutually independence of observations
- Normal distribution of data
- Equal variance in each sample

The normality of the data was evaluated using the Kolmogorov-Smirnov test. The homogeneity of variances was evaluated with the Levene`s test.

The cytotoxicity was assessed by measuring the percentage viable cells in each square in the counting chamber. Cytotoxicity was evaluated in three separate experiments. The data from each sample were compared using an univariate ANOVA in a nested model with experiment number as a random factor and exposure as a fixed factor.

The induced DNA damage was measured in two experiments for each compound. Although there is a large dataset from each experiment, since 50 nuclei in each of the three gels were measured, these are only technical replicates, not independent measurements. To perform statistical analysis, one would want at least three independent experiments, but due to time limits, this was not possible. The data are analyzed statistically to show possible trends in the dataset, but should not be thought of as conclusive statements. The data from the comet assay were analysed in a hierarchical model using univariate ANOVA with experimental number and gel number as random factors, and exposure as the fixed factor for the dependent variable %tailDNA. When a significant difference was found at the 0.05-level between the groups, a Dunnetts T3 test was used as a posthoc test. The Dunnett`s test compares the control group with all other groups and adjusts the error rate according to the number of comparisons made. The Dunnett's T3 test is a variant of the Dunnett`s test which is appropriate when the variances are unequal. The analysis was also done for log transformed data. Since the %tail-DNA can have the value 0, a small figure (0.000001) was added to all values prior to log transformation.

The analysis of gene expression was done in two independent experiments with three technical replicates for the housekeeping gene and five technical replicates for Bcrp1. There is some disagreement as to whether the technical replicates should be treated as the experimental

unit or whether one needs three independent experiments before being able to perform a statistical analysis of real-time PCR-data. Due to time limits, it was not possible to perform a third experiment, but a statistical analysis is done nonetheless to look for trends in the data. Data from real-time PCR experiments were analyzed with several methods. To identify the appropriate dilution and to examine amplification efficiencies of the two genes, a logistic regression analysis was done with the results from the dilution experiment as suggested by Livak and Schmittgen (2001). This analysis was done in Microsoft Office Excel 2003.

The ΔCT values from the exposed samples were tested against the control with the Student's t-test as suggested by Yuan *et al.* (2006), using MatLab. Here, the technical replicate is used as the experimental unit and the analysis was done in each experiment separately. To take into consideration that data were compared several times, the p-value for significance was adjusted by the quite conservative Bonferroni method: $\alpha_{new} = \alpha_{old}/n = 0.05/3 = 0.0167$, where n is the number of comparisons made.

Additionally, data were analyzed by subtracting one of the three 18S-CT-values for the sample from each gene-CT-value. Which of the three 18S-CT-values was subtracted was decided by a randomization function in Excel. The resulting ΔCT-values were analyzed by univariate ANOVA. Due to a lack of normality and homoscedasticity, the logtransformed ΔCT-values were also tested. Graphical presentation of gene expression was done by the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

3. Results

Throughout this chapter, results from the different exposures are presented according to the following colour scheme. Cells exposed to

- 6:2 FTOH: blue
- 8:2 FTOH: red
- PFOA: green
- Adult testicular cells exposed to DBCP: pink
- Spermatogonia exposed to DBCP: yellow

3.1 Purity of cell samples

The fraction of spermatogonia in the different cell samples isolated from 5-8 day old rats was evaluated according to lack of vimentin staining. The 5-8 day old rats are prepubertal, so all the germ cells are in the spermatogonia stage. Therefore, the cells that do not stain with vimentin, are considered spermatogonia. The cells isolated from prepubertal rats were found to contain 68% spermatogonia before the selective cultivation on lectin dishes. A time series of different incubation times revealed that the highest percentage of spermatogonia was achieved after one hour of selective cultivation in lectin coated dishes (figure 18). After one hour incubation, the cells in suspension consist of approximately 82% spermatogonia. At longer incubation times, spermatogonia were found to attach, too. They attached either to the lectin or to the Sertoli cells already bound to the lectin. This resulted in a lower percentage of spermatogonia in suspension. After 24 hours, the suspension contained 55% spermatogonia and 45% Sertoli and other somatic cells. Samples collected after 24 hours will nonetheless be referred to as “somatic cells”, although only less than half of the cells actually are Sertoli and other somatic cells. Testicular cells isolated from adult rats in this project were analysed by flow cytometry in another project in our lab (data not shown). Theses cell samples were found to consist mainly of haploid cells, i.e. spermatids. The remaining cells consist of equal amounts of diploid and tetraploid cells, i.e. primary and secondary spermatocytes, as well as somatic cells.

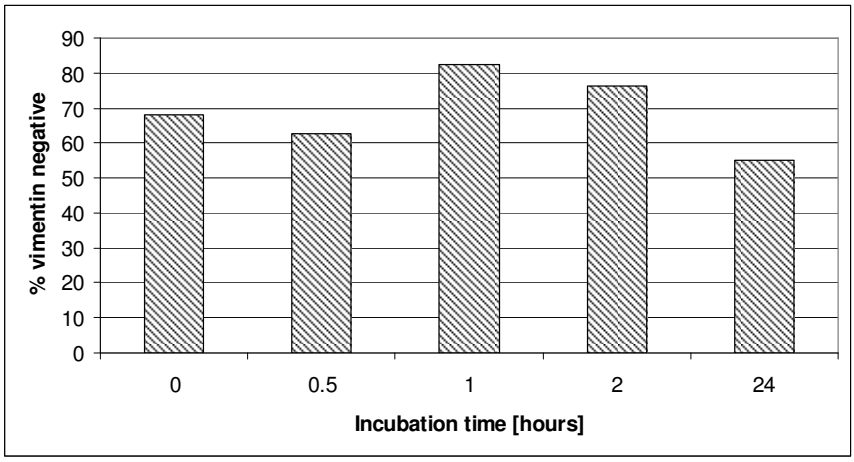


Figure 18: Percentage of vimentin negative cells as a function of incubation time on lectin coated dishes.

3.2 Cytotoxicity

Since all test compounds were diluted in DMSO, the effect of DMSO on cell viability was examined (figure 19). Because this experiment was only performed once, no statistical analysis of the results was done. It appears though, that the exposure to DMSO does not influence cell viability.

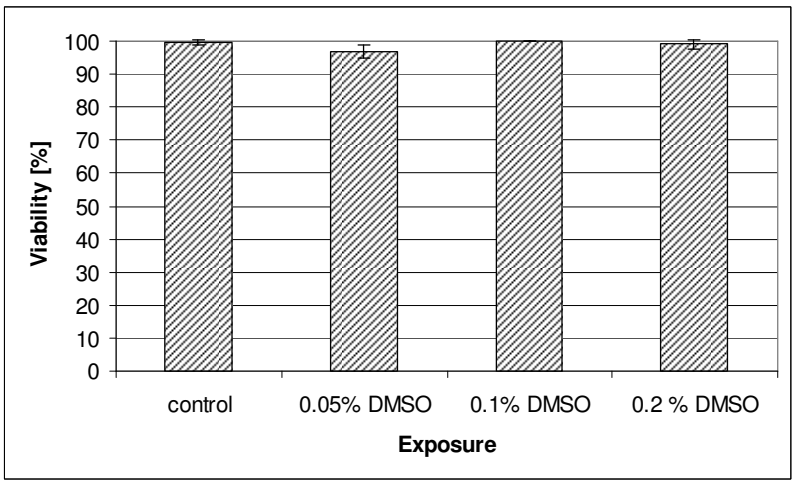


Figure 19: Box plot of viability of testicular cells after one hour of exposure to DMSO. Mean values with standard deviation are shown.

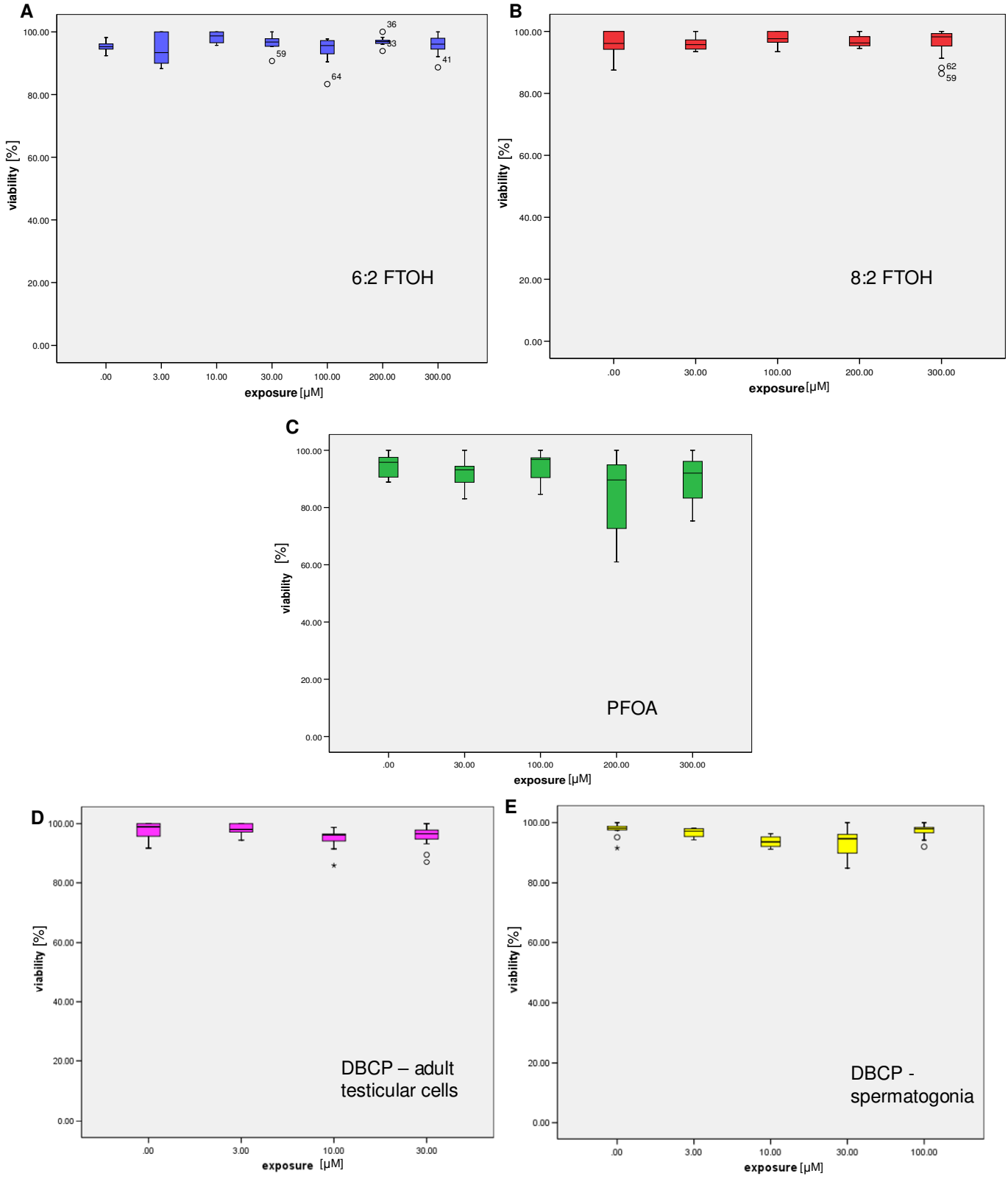


Figure 20: Viability of testicular cells from adult rats exposed to A) 6:2 FTOH (blue), B) 8:2 FTOH (red), C) PFOA (green), D) DBCP (pink) and E) viability of spermatogonia exposed to DBCP (yellow). Results are shown for the three experiments taken together (n=300-400 in each experiment). Shown are median value, interquartiles, outliers (dots) and extreme values (asterisk).

Cytotoxicity was tested in three separate experiments, in which 300-400 cells were evaluated. Viability of cells was not significantly reduced after any of the treatments in either the adult testicular cells or the spermatogonia (figure 20 and table 5). The results were analysed in a hierarchical (nested) analysis with experiment number as a random factor. The data were normally distributed, but the variances were not homogenous. There was no significant difference between the three experimental runs.

Table 5: p-values from the univariate analysis of variance for the viability of cells after exposure to 6:2 FTOH, 8:2 FTOH, PFOA or DBCP.

Substance	**p-value**
6:2 FTOH	0.42
8:2 FTOH	0.62
PFOA	0.45
DBCP (adult testicular cells)	0.30
DBCP (spermatogonia)	0.64

However, the interaction exposure*experiment-number was significant for the adult testicular cells exposed to DBCP, suggesting a difference between the experimental runs. To examine this in more depth, each experiment was analysed alone, and a significant decrease in viability was found in one of the three experiments (p=0.252, p=0.008 and p=0.370 for the three experimental runs, univariate ANOVA). A post hoc test of results from experiment number 2 showed significant decrease in the 10 µM group (p=0.049, Dunnett`s T3) (figure 21). Since only one out of the three experiments showed reduction in cell viability after DBCP exposure at only one concentration, it is assumed that DBCP does not actually impair cell viability at the tested concentrations.

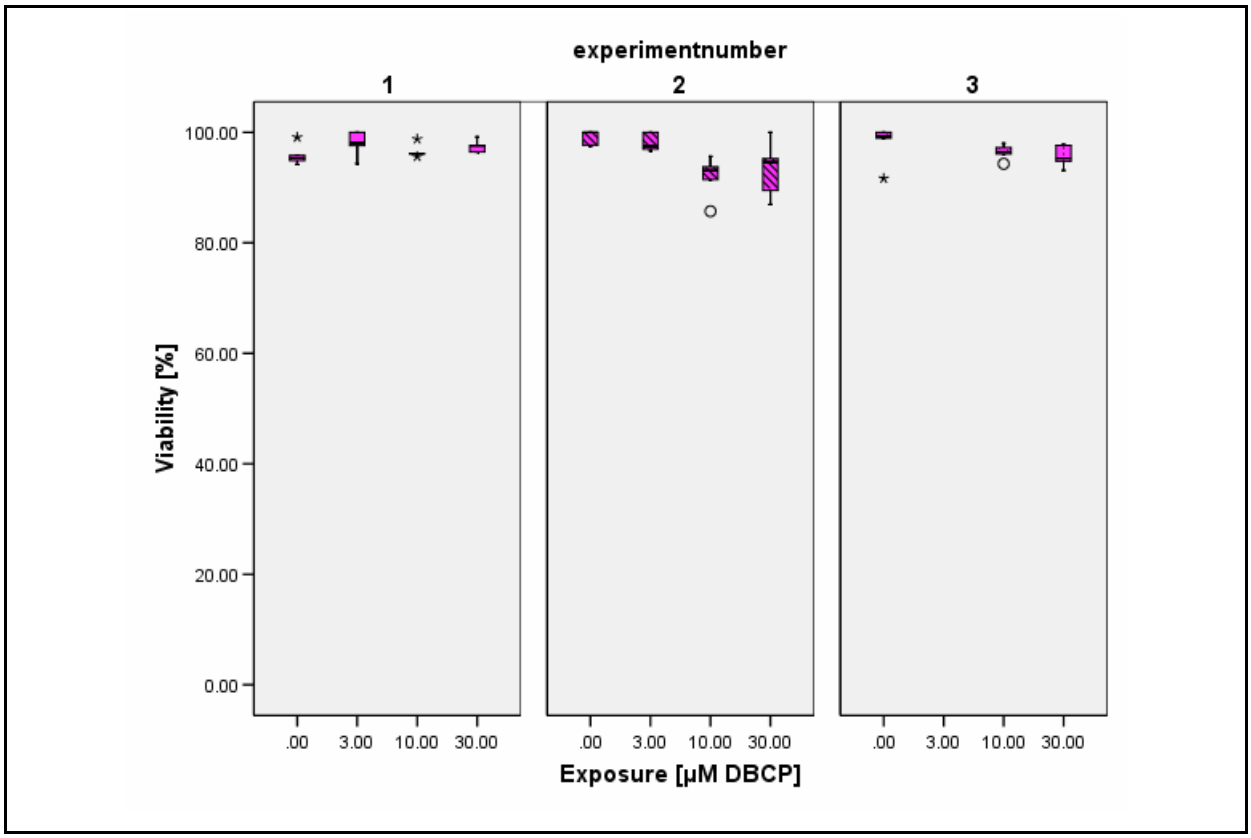


Figure 21: Viability in adult testicular cells after exposure to DBCP. Results from the three experiments are shown. The box plot shows the minimum, first quartile, median, third quartile and maximum of all measurements from the two experiments, as well as outliers.

3.3 DNA damage

In this study DNA damage was measured using the comet assay, which detects single strand breaks, alkalilabile sites, and, due to inclusion of Fpg, oxidized purines. The data obtained by this method are not normally distributed and failed the normality test (Kolmogrow-Smirnov), as well as the test of homoscedasticity (Levene`s test). A logtransformation of the data was attempted, but did lead to neither normality nor homoscedasticity. The results of this approach together with QQ-plots of some data, can be found in appendix 7.5.1 and 7.5.3.

The potential of DMSO to induce DNA damage was assessed to establish whether that the vehicle was responsible for significant DNA damage during exposure. Cells treated with DMSO did not have measurable increased DNA damage (figure 22). The difference in gels treated with Fpg was borderline significant (p=0.07) while the difference caused by DMSO in gels without Fpg treatment was far from significant (p=0.45 from a one-way ANOVA). An effect of DMSO on the measured DNA damage was therefore excluded.

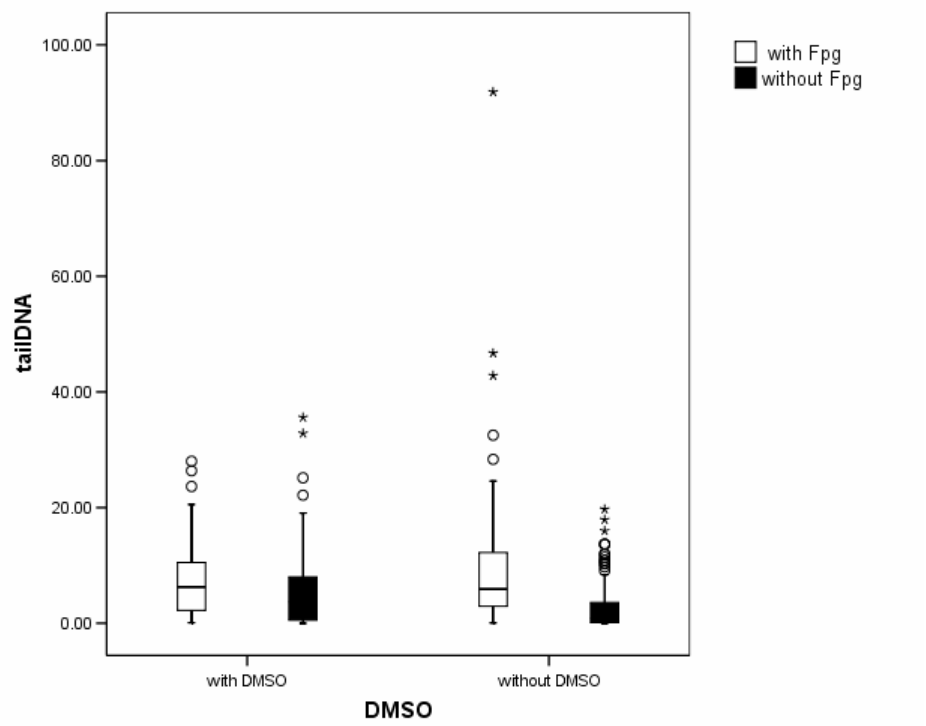


Figure 22: DNA damage in cells exposed to DMSO. Results are shown for both gels with and without Fpg treatment. The box plot shows the minimum, first quartile, median, third quartile and maximum of all measurements from the two experiments, as well as outliers and extreme values.

3.3.1 Calibration of the comet assay by X-ray

Cells were exposed to X-ray to calibrate results from the comet assay and to investigate the efficiency of the comet assay to detect DNA damage in the different cells types. Adult testicular cells, spermatogonia, and somatic cells were exposed to 0, 3, 6, or 10 Gy and the induced DNA damage was measured with the comet assay. Exposure to X-ray induced comparable amounts of DNA damage in spermatogonia, adult testicular cells, and somatic cells, suggesting that the efficiency for detecting DNA damage is similar in those cell types (figure 23). As mentioned earlier, 1 Gy is expected to cause approximately 1000 DNA breaks in a diploid cell. In all cell types 3 Gy induced ~18 %tail-DNA, resulting in 166 breaks per %tail-DNA. A dose of 6 Gy led to ~30 %tail-DNA, which equals 200 breaks per %tail-DNA, while 10 Gy led to ~48%tail-DNA, implying 208 breaks per %tail-DNA. The mean-value of these three doses is 191 breaks/%tail-DNA.

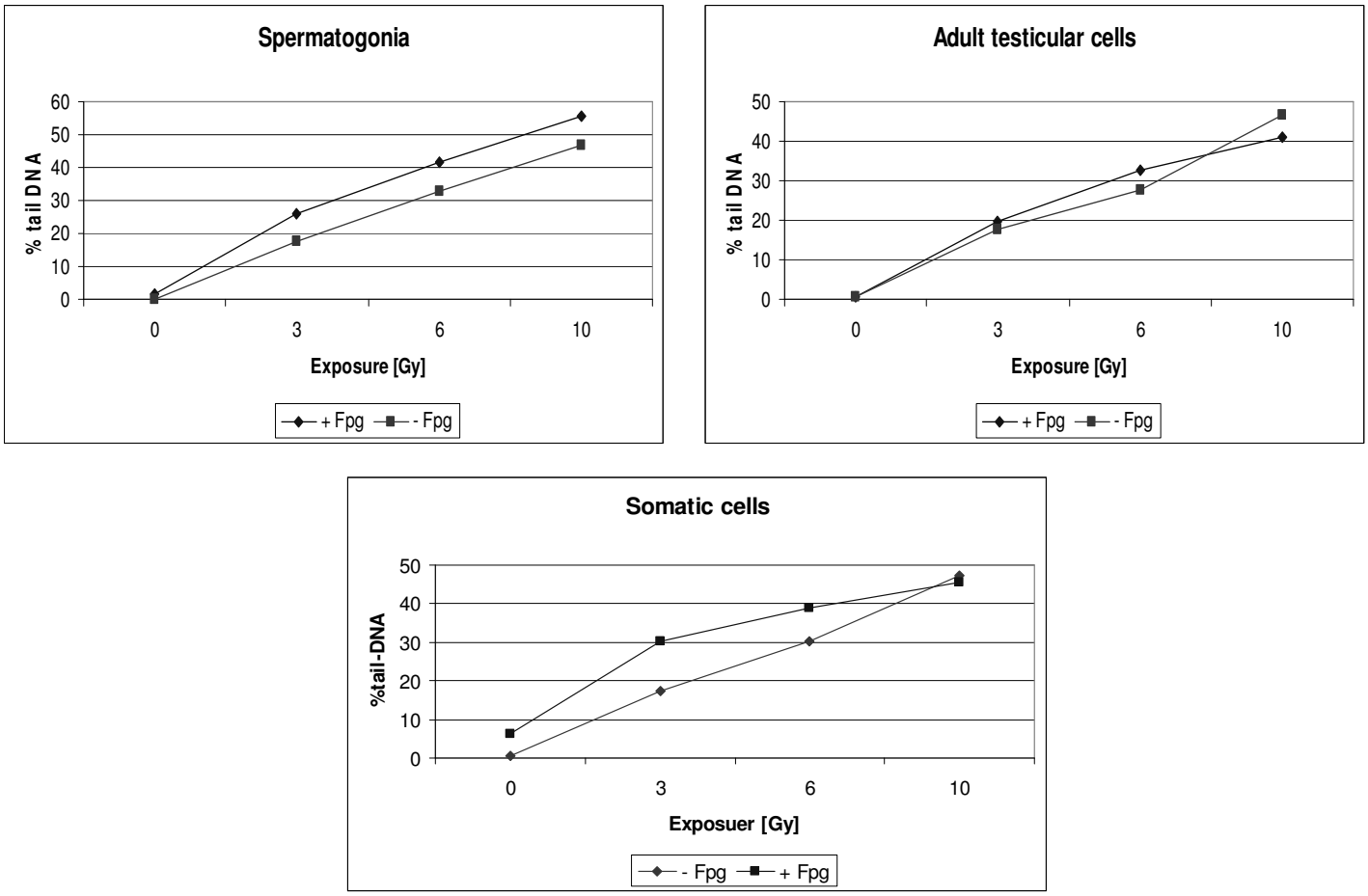


Figure 23: DNA damage in spermatogonia, adult testicular cells, and somatic cells after X-ray exposure. The means of the three medians from each gel are shown.

3.3.2 Polyfluorinated compounds

The amount of induced DNA damage in testicular cells from adult rats after exposure to 6:2 FTOH, 8:2 FTOH, and PFOA was examined. No marked trend in DNA damage was observed. Figure 24 shows the %tail-DNA as a function of exposure to the different polyfluorinated substances. The DNA damage was found to be slightly higher in cells treated with Fpg compared to cells not receiving Fpg-treatment. Figure 24 shows only values for the cells treated with Fpg, while data from cells without Fpg-treatment can be found in the appendix 7.5.2

The %tail-DNA was not significantly increased in testicular cells from adult rats after exposure to 6:2 FTOH, 8:2 FTOH, or PFOA, at concentrations up to 300 μM (table 6). However, as can be seen in table 6, the observed power of the statistical analysis was low. A power >0.8 is normally considered adequate. The lower power observed in this *post hoc* power analysis indicates that one might not reject a false null hypothesis. In addition, there was great variation in %tail-DNA values with many outliers, as can be seen in figure 24. Nonetheless, it can be assumed that no measurable DNA damage was induced in the testicular cells after one hour of exposure to the polyfluorinated substances.

Table 6: F- and p-values from the nested univariate ANOVA of %tail-DNA of cells exposed to 6:2 FTOH, 8:2 FTOH or PFOA, together with the observed power.

Compound	p-value	F-value	Observed power
6:2 FTOH	0.77	3.480	0.566
8:2 FTOH	0.549	1.221	0.088
PFOA	0.329	2.548	0.147

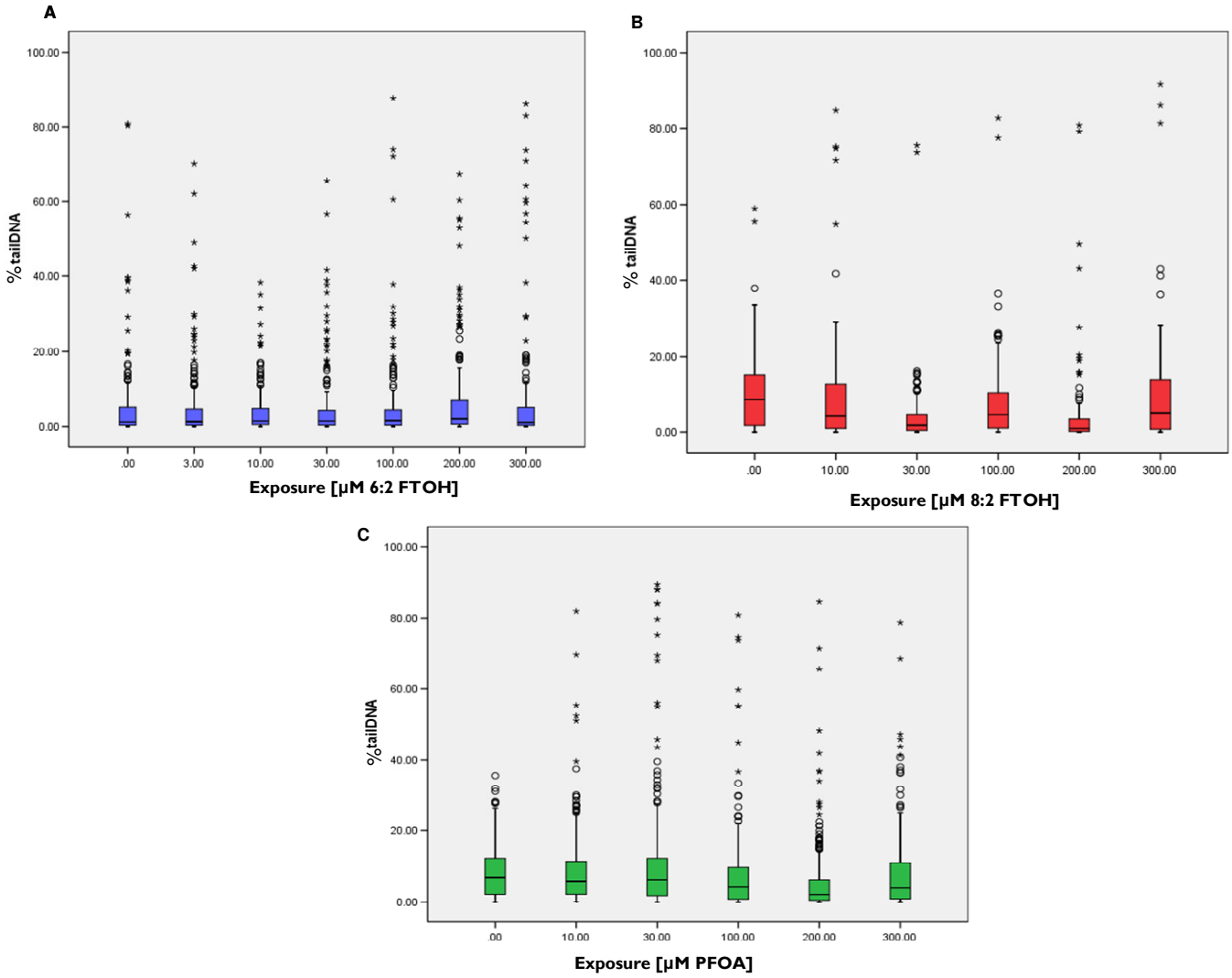


Figure 24: DNA damage as expressed by %tailDNA in adult testicular cells exposed to A) 6:2 FTOH (blue), B) 8:2 FTOH (red), and C) PFOA (green). No significant increase in %tail-DNA was observed. Shown are values with Fpg-treatment. The box plot shows the minimum, first quartile, median, third quartile and maximum of all measurements from the two experiments. The circles represents outliers (> 1.5 x IQR) and asterix (*) represent extreme points (> 3 x IQR).

3.3.3 Dibromochloropropane (DBCP)

The induction of DNA damage following DBCP exposure was investigated in three cell types: adult testicular cells, spermatogonia and somatic cells. The highest level of DNA damage was found in somatic cells, followed by adult testicular cells. Spermatogonia showed the least DNA damage (figure 25).

One of the goals was to examine the susceptibility to DNA damage induced by DBCP in the different testicular cell types. As mentioned before, the somatic cell samples are composed of 45% Sertoli cells and 55% spermatogonia. The results obtained in the comet assay were used as an indication of the susceptibility of Sertoli cells, but due to the poor purity not analyzed statistically. The results show a trend towards higher susceptibility of Sertoli cells to DBCP compared to both adult testicular cells and spermatogonia (figure 25).

Data from adult testicular cells and spermatogonia were analyzed statistically. The %tail-DNA was significantly increased in both adult testicular cells ($F=86.849$, $p<0.001$, univariate ANOVA) and in spermatogonia ($F=90.652$, $p<0.001$, univariate ANOVA). The post hoc test showed that all the DNA damage was increased at all concentrations relative to the control ($p<0.001$, Dunnett`s T3), except for adult testicular cells exposed to 3 μM DBCP ($p=0.122$, Dunnett`s T3). Therefore, a fit of the data to a dose-response model was attempted. The models with the best fit can be found in the appendix. However, due to poor fit to the models ($R^2<0.5$), this approach was abolished. For graphic representation of the models, refer to appendix 7.5.4.

The level of induced DNA damage was generally lower in spermatogonia compared to adult testicular cells. This difference was especially marked in cells not treated with Fpg, where the response at both 10 μM and 30 μM was significantly different in the two cell types ($p<0.001$). Exposure to 30 μM DBCP led to ~20%tail-DNA (≈3820 DNA breaks/cell) in spermatogonia, while the same concentration induces ~50%tail-DNA (≈ 9550 DNA breaks/cell) in adult testicular cells. DNA damage measured in cells not treated with Fpg shows strand breaks and alkalilabile sites, while Fpg treatment also reveals oxidized purines. The amount of net Fpg-sensitive sites is much higher in spermatogonia, suggesting that spermatogonia are more prone to oxidative DNA damage than adult testicular cells.

Taken together these results suggest that DBCP causes DNA damage in all tested cell samples. Further on, spermatogonia seem to be more resistant to strand breaks and alkalilabile sites caused by DBCP exposure than adult testicular cells, while the sensitivity towards oxidized purines is higher in spermatogonia. The somatic cell population showed the highest susceptibility towards DNA damage.

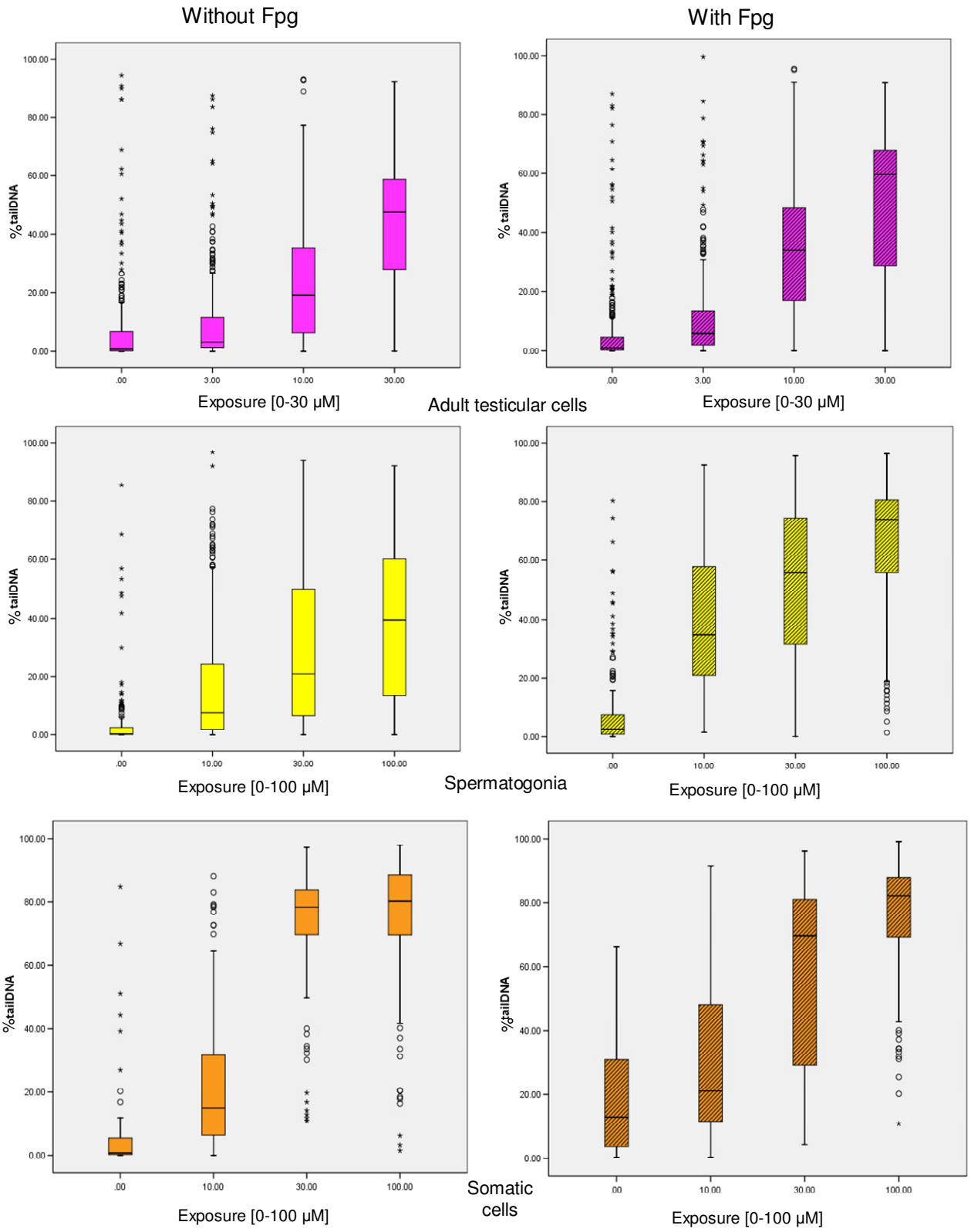


Figure 25: DNA damage as expressed by %tailDNA in adult testicular cells exposed to DBCP (top panel, pink), in spermatogonia (yellow), and in somatic cells (orange, bottom panel). The left column shows values without Fpg treatment, while the right column shows measurements from gels treated with Fpg (striped bars). Note the different exposure concentrations used in adult testicular cells. The box plot shows the minimum, first quartile, median, third quartile and maximum of all measurements from the two experiments, as well as outliers and extreme values.

3.3.4 Repair capacity of DBCP induced DNA damage

The repair capacity was evaluated using the comet assay in two separate experiments for each cell type. Unfortunately, only one experiment per cell type gave satisfactory results, the other showing very high background levels of DNA damage and less DNA damage than expected in treated cells. One of the possible reasons here is the use of an old batch of diluted DBCP which had been frozen and thawed many times, so a new batch was prepared for the second round of experiments, giving better results.

Cells treated with Fpg showed no reduction in %tail-DNA during 24 hours (appendix 7.5.5), suggesting no repair of oxidative DNA damage or constant induction of new oxidative damage. However, cells without Fpg-treatment showed reduction in %tail-DNA, implying that strand breaks and alkalilabile sites were repaired.

No clear difference in repair capacity of DBCP-induced damage was found between adult testicular cells and spermatogonia (figure 26). The repair after an exposure causing approximately 60%tail-DNA (≈11460 breaks/cell) in the cells without Fpg-treatment was analyzed in more depth (adult testicular cells exposed to 30 μM, spermatogonia exposed to 100 μM). During the first 4 hours, spermatogonia reduced the DNA damage as measured in %tail-DNA by 51.8% (repair of 5936 breaks), while adult testicular cells repaired 33.7% (≈3862 breaks). The $t_{1/2}$ for the induced damage, as calculated from a single experiment, was 4 hours for spermatogonia and 8 hours for adult testicular cells. Spermatogonia seemed to be able to repair all damage in the course of 24 hours (repair rate of approximately 11500 breaks per 24 hours), while approximately 18%tail-DNA is still measured in adult testicular cells (repair rate of approximately 8000 breaks per 24 hours). A posthoc test showed significantly more repair in spermatogonia than adult testicular cells after 4 and 24 hours after this DBCP exposure ($p=0.034$ and $p<0.001$, Dunnett`s T3)).

Taken together the results show a slight trend towards faster repair in spermatogonia.

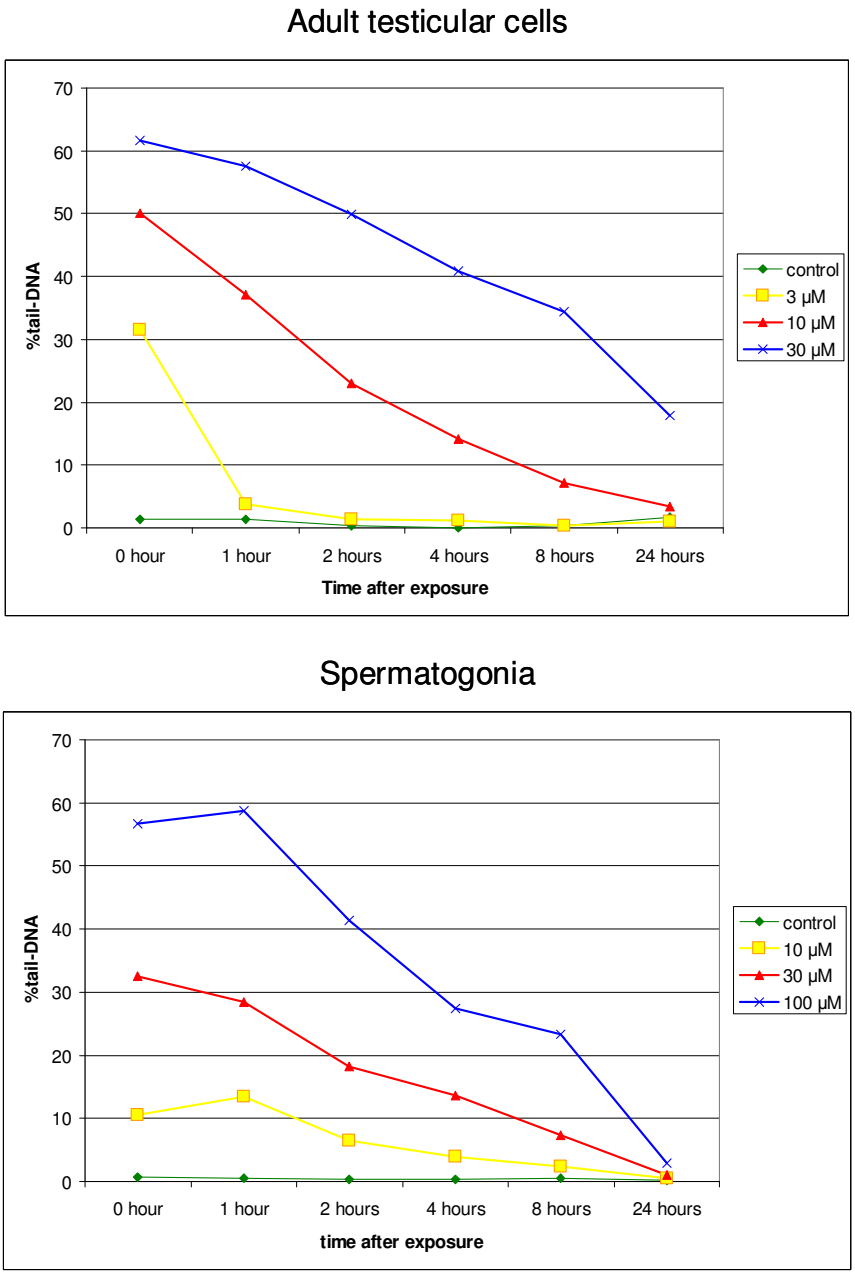


Figure 26: Repair of DNA damage after exposure to DBCP in adult testicular cells and in spermatogonia. The mean of medians from each gel is shown. Cells were not treated with Fpg.

3.4 Gene expression

The gene expression of Bcrp1, Oat2, and P-gp was measured after one hour of exposure to 6:2 FTOH, 8:2 FTOH or PFOA. RNA was isolated from the samples, cDNA was synthesized and real-time PCR was performed.

The amount of RNA varied considerably between samples and was dependent on the amount of cells in the individual sample (figure 27). The average amount was 47.16±31.3 ng/µl. Nevertheless, the amount of cDNA synthesized from the samples was more similar with an average amount of cDNA in the samples of 2297±279ng/µl. The purity of the RNA samples was 1.6 for the 260/230 ratio and 2.1 for the 260/280 ratio. A 260/280 ratio of ~2.0 is considered pure RNA, while the 260/230 ratio should be between, 2.0 and 2.2. The 260/280 ratio of the samples was close to the desired value, while the 260/230 ratio was lower, which can indicate the presence of contaminants absorbing at 230.

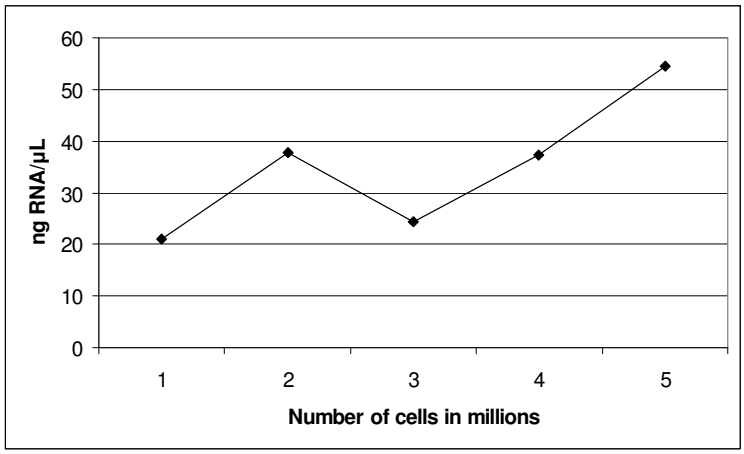


Figure 27: Amount of RNA extracted as a function of number of cells in the sample.

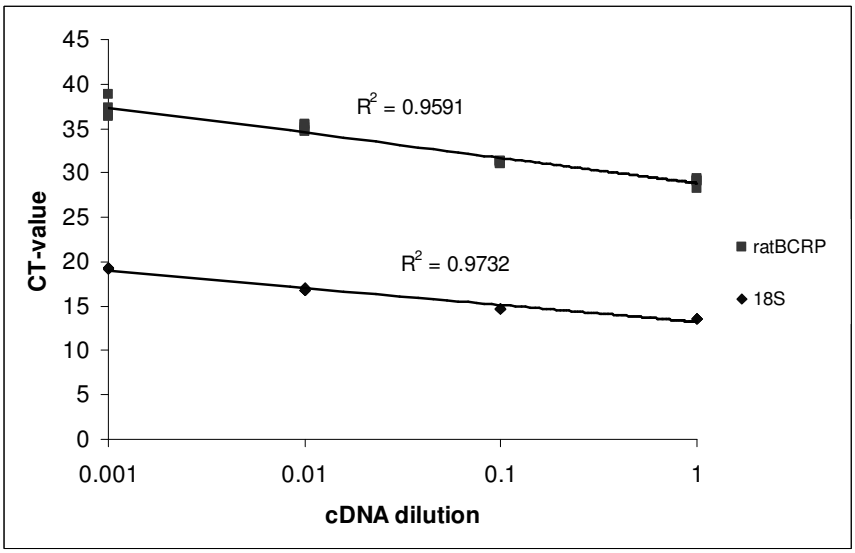


Figure 28: Regression analysis of log cDNA dilution versus CT-value. Shown are the CT-values for the 5 replicates of each gene (dots) as well as the regression line with the coefficient of determination (R^2). The trend line for 18 S is y =-0,8307Ln(x) + 13,224, while the formula for the Bcrp1 trend line is y = -1,2318Ln(x) + 28,842. The high R^2 suggests high agreement between single measurements. Similar slopes of the two trend lines imply equal amplification efficiency.

Three genes were analysed using the real-time PCR. However, primers for two of the genes (Oat2 and P-gp) did not give a satisfactory result in the real-time PCR, although several primers were tested. A high CT-value and high levels of unspecific products resulted in the decision not to use the data obtained with Oat2 and P-gp primers. Only the expression of Bcrp1 is therefore analysed further.

The linear regression analysis of log cDNA dilution versus CT-value is shown in figure 28. The high R^2-value reveals that the linear regression is a good model for the dilution series, explaining 95 and 97% of the variation for Bcrp1 and 18S, respectively. It also shows that the technical replicates are very alike each other. Additionally, the analysis is used to find the best dilution to be used in the real-time PCR. A high CT-value leads to a lot of unspecific background, as well as unspecific products during the real-time, so a 0.1 dilution should be used, since 0.01 dilution gives a CT-value over 35 for Bcrp1. As previously described, the $2^{-\Delta\Delta CT}$-method assumes equal amplification efficiencies of the housekeeping gene and the target gene. A similar slope of the dilution curves for 18S and Bcrp1 implies that this assumption is valid.

Taken together the results from the regression analysis suggest the use of a 0.1 dilution of the cDNA, and it supports the use of the $2^{-\Delta\Delta CT}$-method.

The expression of 18S was reasonably stable in the different sample (figure 29), indicating no effect of the test compounds on expression of the housekeeping gene.

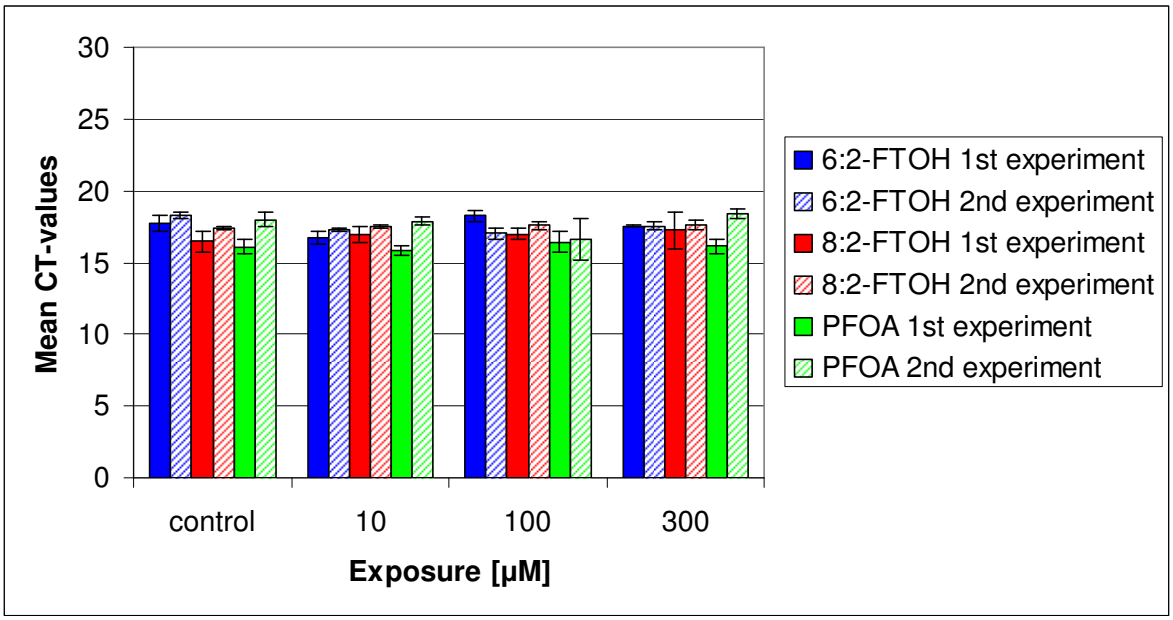


Figure 29: Variation in CT-value for the housekeeping gene 18S in the different samples. The mean value from the three samples with standard derivation is shown.

Gene expression data for Bcrp1 were analysed statistically by two different methods, since neither method seemed to be flawless. The results from the Student's t-test can be found in

table 7, while results from the second analysis can be found in appendix 7.5.6 (raw data, as well as log transformed data). Exposure to PFOA did not lead to significant changes in gene expression (figure 30). However, a significant difference from the control of two samples exposed to 6:2 FTOH (10 and 100 µM 6:2 FTOH) was observed in one of the experiments, where the fold change was reduced to 0.31 and 0.28, respectively. The second run of this experiment showed no significant difference between controls and exposed. Exposure to 8:2 FTOH lead to a reduction in fold change to 0.52 and 0.40, for exposure to 10 µM and 100 µM 8:2 FTOH respectively. This was significant as tested by the Student's t-test in one experiment. The same exposure was significant in the second statistical approach (p=0.027 for univariate ANOVA), but the post hoc test showed no significant reduction (p=0.307 and p= 0.917 for Dunnett`s test). The second experiment did not show significant reduction of gene expression after exposure to 8:2 FTOH.

Taken together, the results indicate no clear trend of the expression of Bcrp1 after one hour exposure to PFOA, 6:2 FTOH or 8:2 FTOH. However, there was considerable variability between the experiments, as can be seen in figure 31. Differences between experimental runs is a commonly observed feature of the real-time PCR due to the high sensitivity of the method combined with technical errors.

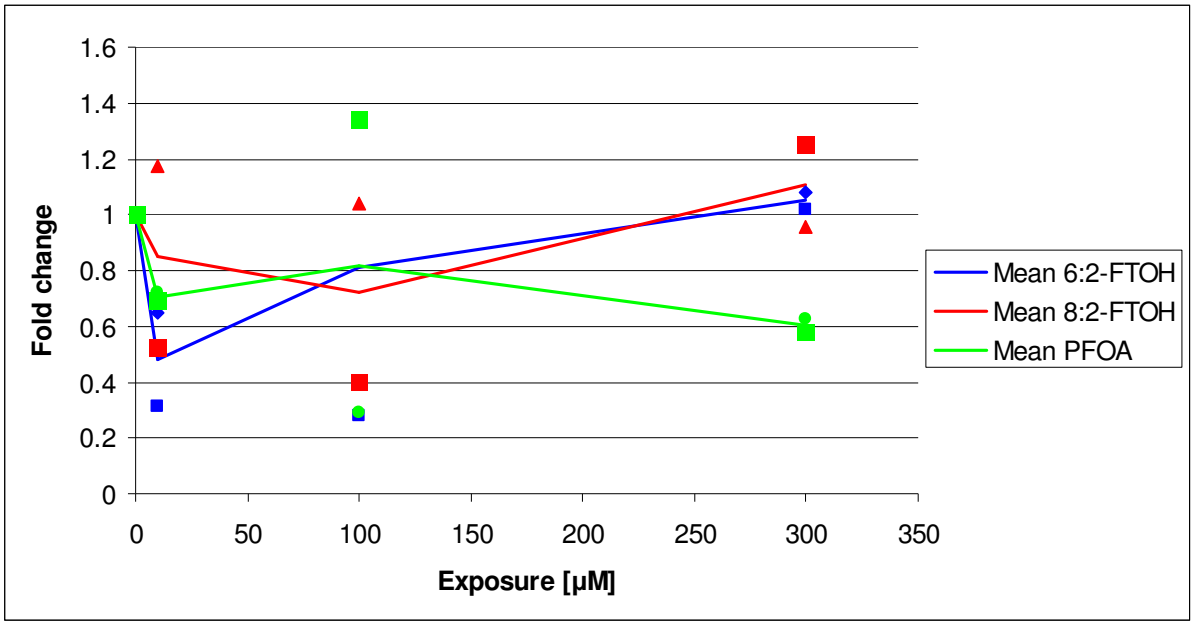


Figure 30: Change in expression of Bcrp1 as a function of exposure. The mean $2^{-\Delta\Delta CT}$-values from both experimental runs are shown (dots) and the average of these two experiments. Blue dots represent 6:2 FTOH, red dots 8:2 FTOH and green dots PFOA.

Table 7: Results from the Student's t-test (df=5) of ΔCT-values from exposed samples versus control samples. The Fold change is also shown. P-values <0.0167 are considered significant and marked by an asterisk.

Exposure		Dilution of cDNA	Fold change ($2^{-\Delta\Delta CT}$)	p-value
6:2 FTOH	10 µM	1:10	0.31	0.116
	100 µM	1:10	0.28	0.355
	300 µM	1:10	1.01	0.718
	10 µM	1:100	0.64	0.00004 *
	100 µM	1:100	1.33	0.0003 *
	300 µM	1:100	1.08	0.911
8:2 FTOH	10 µM	1:10	0.52	0.769
	100 µM	1:10	0.40	0.911
	300 µM	1:10	1.25	0.931
	10 µM	1:100	1.17	0.0029 *
	100 µM	1:100	1.03	0.004 *
	300 µM	1:100	0.95	0.228
PFOA	10 µM	1:10	0.72	0.156
	100 µM	1:10	0.28	0.424
	300 µM	1:10	0.62	0.0902
	10 µM	1:100	0.69	0.307
	100 µM	1:100	1.34	0.071
	300 µM	1:100	0.58	0.208

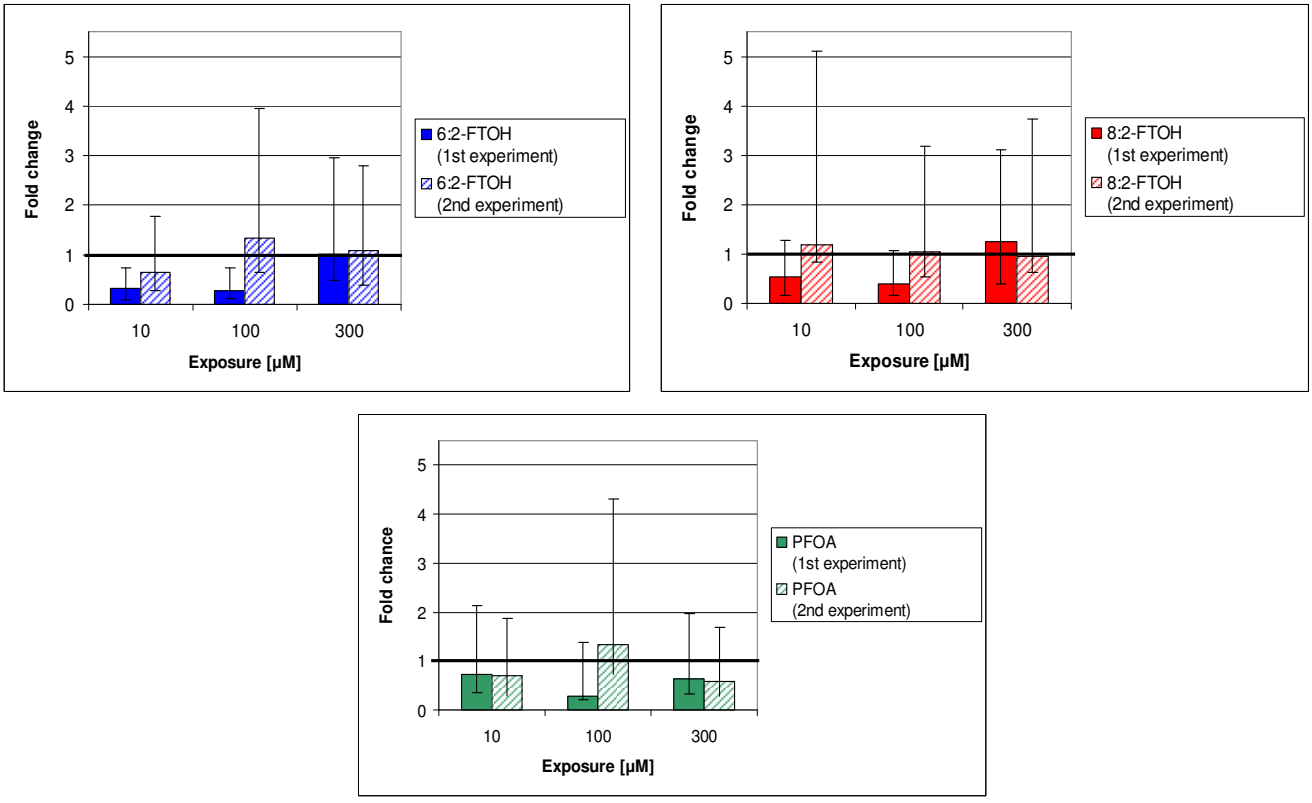


Figure 31: Alteration in expression of Bcrp1, expressed as $2^{-\Delta\Delta CT}$-value, after exposure to 6:2 FTOH, 8:2 FTOH or PFOA. Results from two separate experiments with five technical replicates in each are shown. The bars represent mean $2^{-\Delta\Delta CT}$-values with standard deviation. A fold change value of 1 implies no change in gene expression relative to the control. A lower value suggests reduction in gene expression, while a value above 1 implies induction of the gene.

4. Discussion

4.1 Experimental design

Extrapolation from *in vitro* experiments to human toxicity

In vitro systems cannot replicate the complex interactions of animals *in vivo*, but they can provide important predictive information about the biological activity of a compound. Yet, one has to keep in mind that primary cell cultures prepared from one animal have limited probative force/conclusiveness since they only represent the reaction of one individual from one species. In the experiments presented here, cells were always pooled from at least two animals, somewhat increasing the predictive power of the results. Yet, due to this design, some information about individual variation is lost. Primary cell cultures maintain many of their *in vivo* characteristics and can therefore be more appropriate for extrapolating to humans than cell lines. But care still has to be taken to interpret these data, especially when extrapolating from one species to another (here, rats to humans). The extrapolation from laboratory experiments to humans includes also an extrapolation from very high doses/concentrations used in experiments to low levels of the test compound found in humans. In this study, cells are exposed to PFOA at concentrations up to 300 μM, while typical levels in human blood samples are 0.01 μM. Additionally, it is important to not only know the mechanism of toxicity of a compound, but also the shape of the dose-response curve in order to be able to extrapolate correctly.

As described earlier, both PFCs and DBCP show very dissimilar responses in different species. The main differences in response to PFCs are the vast differences in elimination half-lifes and differences with respect to PPARα-induction. The much longer half-life in humans makes PFCs potentially more hazardous to humans, while the lack of response to PPARα-inducers in humans suggest a lower susceptibility. Vast differences regarding the response in different species have also been reported for the effects of DBCP; some species, like the hamster, show almost no susceptibility towards DBCPs effect on testicular cells, while others are highly susceptible. The induced testicular damage has previously been shown to be related to DNA damage induced *in vitro* in the different species. *In vitro* exposure of human testicular cells showed hardly any measurable induction of DNA damage (Bjørge *et al.*, 1995). These large species differences make extrapolation less precise, and sometimes the extrapolation may even be misleading.

False positive and false negative results

A false positive effect can be observed when the vehicle used gives a positive response in the assay. All tested compounds were dissolved in DMSO. The effects of DMSO on cytotoxicity and the induction of DNA damage were therefore tested. No cytotoxic effect was found at the used concentrations, and no measurable DNA damage was induced.

Additionally, false positive results in the comet assay can be due to DNA degradation resulting from cell death, and thereby a secondary effect of cytotoxic effects of a compound, in contrast to a genotoxic effect. Viability of cells was therefore monitored prior to the comet assay, and was always found to be >90%. It is therefore unlikely that cytotoxicity caused false positive results in the comet assay.

False negative results can possibly be obtained due to short exposure times. Both cytotoxicity and induction of DNA damage can occur only after prolonged exposures, but especially the investigation of changes in gene expression is dependent on either long exposure times or some post exposure time. Primary cell cultures of testicular cells in suspension are prone to both false negative and false positive results due to the suboptimal culture conditions, especially during prolonged culturing. One way of avoiding this could be *in situ* incubation of the cells in seminiferous tubules. Here, the supporting tissue and especially Sertoli cells are present, which has been shown to reduce apoptosis and increase the repair rate of DNA damage (Bentley and Working, 1988b; Bentley and Working, 1988a).

A false negative result can be obtained if the tested compound is not stable under experimental conditions. Both fluorotelomer alcohols (FTOHs) are volatile compounds and some loss of the compounds by evaporation during exposure cannot be excluded. Unfortunately, no measurement of exposure concentration could be done at the end of exposure.

Another possible reason for a false negative result in the comet assay is a selection of undamaged cells. It is conceivable that some cells are heavily damaged, which can lead to apoptosis or necrosis. These cells can be lost during washing steps and are therefore possibly not seen in the comet assay. This could have been controlled for by measuring the number of cells observed in the comet assay at the various exposure concentrations. Alternatively one could monitor the amount of DNA in the supernatant after centrifugation. An increase in DNA amount in the supernatant would be due to an increase in dead cells and thus selection of undamaged cells.

Statistical analysis of comet data

All experiments using the comet assay were performed twice. To be able to perform a proper statistical analysis, results from three independent experimental runs are necessary. Due to time constraints, it was not possible to perform a third experiment. Nonetheless, the data obtained in the two experimental runs are analyzed statistically to look for trends.

Data obtained by the comet method are usually not normally distributed. Also in this study, all comet data failed the normality test, as well as the test for homogeneity of variance. These findings suggest the use of non-parametric tests. The usefulness of non-parametric tests (Kruskal-Wallis and Mann-Whitney test) was investigated by Duez *et al.* (2003). These authors considered the single cell to be the experimental unit and found non-parametric methods to be overly sensitive, detecting significant, but objectively unimportant, differences. However, this might be due to the erroneous use of the single comet as the experimental unit, resulting in a very large n (n=100).

The experimental design is nested and non-parametric tests can not be used in nested analysis. A possibility would have been to analyse the data with a parametric analysis of median values obtained in each gel. This would bring about a loss of information, as experiment and gel number can not be factors in this analysis. Also, the power of this analysis would be quite low, since n=6. In addition, the distribution of the data is different in control and treatment groups: data obtained from gels in which cells have little DNA damage are right skewed, while data from gels with highly damaged cells are left skewed. Non-parametric tests do not require normality, but they require similar distribution in all groups. There is however some discussion regarding how strict this requirement is (Zar, 1996).

Another possibility is transformation of data using various functions, which sometimes can increase normality and homogeneity of variances. In this study log transformation was tested and no improvement of the data quality with respect to normality or homogeneity was found.

ANOVA is thought to be a fairly robust method, so even if some of the underlying assumptions (independence of observations, normality, and homoscedasticity) are not fulfilled, it might still be used. It is assumed that it can be conducted when the variances differ by a factor of two and where normality is a minor violation (van Belle, 2002). The variances between samples were found to differ by a factor of up to 5 in this study, and data were often far from normal as detected by the Kolmogorov-Smirnov test. This test is a goodness-of-fit statistics which can be overly sensitive when the sample size is large because real data are unlikely to be perfectly distributed (Conover, 1980). In addition, there are 150 data points per exposure and experiment, making the central limit theorem applicable: the means of a random

sample will be normally distributed as the sample size increases, although the original data is not normally distributed, since the sum of independent samples from any distribution with finite mean and variance converges to the normal distribution as the sample size goes to infinity (Zar, 1996).

For reasons mentioned here, statistical analysis of comet data is challenging. As Lovell put it: "There is unlikely to be a single correct statistical analysis for all designs, but there are a number of potentially wrong analyses" (Lovell and Omori, 2008). The nested ANOVA was chosen in this study as the best method available. However, one must not forget that the underlying assumptions, homogeneity of variance and normality, are violated and experiments were only performed twice, so care has to be taken when analysing the results of the ANOVA. The heteroscedaticity (heterogeneity in variance) of the data results in a lower power of the tests and thus a lower probability of rejecting a false null hypothesis.

4.2 Polyfluorinated compounds

The toxicity of 6:2 FTOH, 8:2 FTOH, and PFOA was tested in adult testicular cells from rats. These cells consist primarily of spermatids; the remaining cells are spermatocytes, spermatogonia and Sertoli cells.

4.2.1 Cytotoxicity

Cytotoxicity was evaluated by the Trypan Blue exclusion test. Viability was not reduced after one hour of exposure to 6:2 FTOH, 8:2 FTOH or PFOA at concentrations up to 300 μM. This is in compliance with results by Martin *et al.* (2005) who found no increased cell mortality after exposure to either 8:2 FTOH or 6:2 FTOH at concentrations up to 200 μM for 4 hours (Martin *et al.*, 2005). The same conclusion was reported by Liu *et al.* (2007): Exposure to 6:2 or 8:2 FTOH at concentrations up to 1.1×10^{-5}M (11 μM) for 44 hours did not reduce cell viability in primary cultured *Tilapia* hepatocytes (Liu *et al.*, 2007a). However, 200 μM PFOA for 24 hours led to apoptosis in the human hepatoblastoma HepG2 cells (Mulkiewicz *et al.*, 2007). Although we observed no cytotoxicity after one hour of exposure, a cytotoxic effect of the compounds after prolonged exposure cannot be excluded.

In addition, the sensitivity of the Trypan Blue Exclusion test is debatable. Storer *et al.* (1996) suggest that the Trypan Blue exclusion test is not an appropriate method for detecting cytotoxicity, since loss of membrane integrity can be a late event in toxic cell death. The authors suggested the use of a delayed Trypan Blue exclusion test 3 hours after the exposure. Others suggest that the Trypan Blue exclusion test is overly sensitive since it measures

membrane instability, which can be due to other causes than chemical induced cytotoxicity, for instance excessive pipetting or centrifugation (Collins *et al.*, 2008). Another method that could have been used here is the staining with propidium iodide (PI) and Hoechst in order to distinguish between apoptosis and necrosis of exposed cells. This method is more sensitive than the Trypan Blue exclusion test. Additionally, the MTT assay could have been used

4.2.2 DNA damage

The induction of DNA damage in adult testicular cells was evaluated using the comet assay. Neither 6:2 FTOH, 8:2 FTOH nor PFOA led to a significant increase in DNA damage as measured by the Comet assay after one hour of exposure to concentrations up to 300 µM. However, the observed power of the applied statistical test was low, as previously discussed. This can lead to not rejecting a false null hypothesis. In this case the result from the statistical analysis can therefore lead to the conclusion that the PFCs tested did not induce DNA damage although they actually do. Nonetheless, unexposed cells are expected to have 5-15%tail-DNA. None of the treatments led to a median value above 15%tail-DNA. The comet assay is a very sensitive assay for detecting strand breaks and alkalilabile sites. When including the repair enzyme Fpg, ring-opened purines and 8-oxoguanine, and possibly some small adducts, are detected in addition to single strand breaks. Since %tail-DNA did not increase in cells exposed to 6:2 FTOH, 8:2 FTOH, or PFOA, it can be assumed that the test compounds did not induce significant numbers of single strand breaks, AP sites, ring-opened purines or 8-oxoguanine during this short *in vitro* exposure.

These results are in compliance with findings by Stankowski (2001) who showed that 8:2 FTOH was not mutagenic in the *Salmonelle/Escherichia coli* mutation assay, as well as findings by Freire et al. (2008) who reported no mutagenic effect of PFOA. Takagi *et al.* (1991) found an increase of 8-hydroxydeoxyguanosine (8-OH-dG) in the liver of rats after exposure to PFOA. However, the authors argue that this was a secondary effect of the peroxisomal proliferation caused in the liver and not a direct oxidizing effect of PFOA. Panaretakis *et al.* (2001) suggest that PFOA can induce oxidative stress by disrupting the inner membrane of the mitochondria. This suggests that PFOA exposure could lead to increased oxidative DNA damage. However, this was not observed in this study.

It has to be kept in mind, though, that cells were only exposed for one hour. The expert panel for developing guidelines for the use of the comet assay in genetic toxicology suggest an exposure period of 3 to 6 hours (Tice *et al.*, 2000), and it is possible that an effect on the DNA could have been observed after longer exposure times.

In some of the experiments, a possible negative trend of %tail-DNA with increasing exposure was seen (see appendix 7.5.2, PFOA). This was not significant, but can possibly

suggest a DNA cross-linking effect of the compounds or an inhibition of repair enzymes, leading to a lower incision rate. Cross-linking is expected to be caused by agents with two reactive groups, for instance two epoxides. Neither the FTOHs, nor PFOA is expected to have this property. Because of this, coupled with time constraints, the possible cross-linking effect was not further addressed in this study. Another explanation of reduced %tail-DNA with increased exposure is an inhibition of repair enzymes such as DNA-glycosylases. These enzymes induce incision-related breaks as the initial step of DNA repair, which will appear as single strand breaks in the comet assay. An inhibition of these enzymes would lead to higher levels of DNA damage as 8-oxoG, but less incision-related breaks. Vanden Heuvel *et al.* (1992) suggested that PFOA binds to sulfhydryl groups of proteins. This is a common mechanism of enzyme inhibition, supporting the theory that PFOA might inhibit DNA repair enzymes. However, at the tested concentrations the effects were very small, so this is most likely not an important toxicity mechanism for PFOA. To test whether PFOA actually does inhibit repair enzymes a known amount of DNA damage could be induced by exposure to another chemical, UV- or X-ray, followed by incubation with PFOA. If PFOA actually inhibits repair enzymes, a reduced repair rate should be observed in cells with high PFOA exposure.

It must also be remembered that the comet assay can be used to show genotoxicity of a compound, but cannot demonstrate the lack of genotoxicity, since not all types of DNA lesions can be detected in a standard comet assay. For instance, bulky adducts are not measured in the standard comet assay. It is however possible to observe these and other lesions by inhibiting DNA polymerase or ligase, which leads to the accumulation of single strand breaks (SSBs) formed as an intermediate step in the repair of DNA lesions. The SSBs can then be measured in the comet assay. Cytosine-1-β-D-arabinoduranoside (AraC) or aphidicolin could have been used in these co-exposure experiments (Friedberg *et al.*, 2006). However, this method can only be used in cell types with active NER. The cells in the current study have probably little or no efficient NER, making it impossible to detect bulky adducts with the comet assay.

It is in addition unclear whether the compounds were metabolized. A metabolic product of the compounds may be biologically more active. In order to reduce this possible false negative result, cellular genotoxicity tests often require the addition of hepatic S9 from Aroclor 1254-induced rats or another source of metabolic activation.

4.2.3 Gene expression

An inhibition of effluent transporters such as P-gp, Bcrp1 and Oat2 could lead to intracellular accumulation and a higher toxicity of harmful substances, also known as chemosensitization. This is a quite severe feature of a compound since it blocks one of the basic lines of biological defence and reverses natural resistance. Smital and Kurelec (1998) showed an increase in single strand breaks and DNA adducts after exposure to compounds that inhibit efflux pumps. Non-functional effluent pumps at the blood-testis barrier might be especially serious, leading to an increased exposure of maturing germ cells in their most sensitive stages.

Because of the seriousness of intervention with these protective measures and because of previous studies showing an inhibition of P-gp following exposure to PFOA (Stevenson *et al.*2006), the expression of the breast cancer resistance protein (Bcrp1), organic anion transporter 2 (Oat2), and P-glycoprotein (P-gp) in adult testicular cells was assessed using real-time PCR. The suspension with adult testicular cells consists of mainly spermatids. This expression of Bcrp1 has previously been shown on apical side of myoid cells and on the luminal side of the capillary endothelial cells (Bart *et al.*, 2004).

The real-time PCR results for Oat2 and P-gp were not satisfactory due to high amounts of unspecific products formed in some samples, and no product formation in other samples. This can be due to unspecific primers, contamination of RNA, as suggested by the low 260/230 ratio, or low expression in the examined cells. Constituent expression of Oat2 and P-gp in rat testicular cells had been reported previously (Augustine *et al.*, 2005). However, Melaine *et al.* (2002) found no constituent expression of P-gp in mitotic and meiotic germ cells, but only in spermatozoa. Bart *et al.* (2004) report expression of P-gp in endothelial and myoid cells of the testis. The main cell type in the cell suspension obtained from adult rats is spermatids. It is therefore debatable whether low constituent expression of P-gp in the cell sample can explain the unsatisfactory result from the real-time PCR.

The function of the P-gp transporters has been shown to be inhibited after exposure to several PFC, among others PFOA, in mussel cells by Stevenson *et al.* (2006). The inhibition led then to an induction of the P-gp protein as measured by Western blot analysis 48 hours after the exposure. Due to the poor result of the real-time PCR for P-gp, it is unknown whether PFOA or FTOHs influence this pump in testicular cells.

As described earlier, the results from the real-time PCR with Bcrp1 were analyzed by several statistical methods. The stability of the housekeeping gene in all tested samples indicates that the chosen gene (18S) actually is unaffected by the tested compounds, and that it was appropriate as an internal standard. Furthermore, the regression analysis of different cDNA dilutions indicated that the $2^{-\Delta\Delta CT}$-method could be applied. Due to the small sample

size and discussion concerning the best statistical analysis of real-time PCR data, two different methods were tested; Student's t-test and univariate ANOVA. Since both analyses came to the same conclusion in most cases, the results seem reliable.

PFOA did not significantly alter the expression of Bcrp1 after one hour of exposure at concentrations up to 300 μM. The picture is however more complicated for both 6:2 FTOH and 8:2 FTOH. Both statistical analyses showed that two samples from the first experiment exposed to 6:2 FTOH were significantly different from the control: samples exposed to 10 μM and 30 μM 6:2 FTOH had lower expression of Bcrp1. The cells exposed to 300 μM 6:2 FTOH did not show this effect. The second experiment showed no significant differences between control and treatment in either analysis. However, analysis of the second experiment revealed a lack of homoscedasticity, which can lead to type II error (not rejecting a false null hypothesis). Based on data from this study, it is therefore not possible to conclude whether or not 6:2 FTOH leads to downregulation of Bcrp1 in rat testicular cells. With respect to 8:2 FTOH, two of the exposed samples showed significant reduction of Bcrp1 expression when analyzed by the Student's t-test (10 μM and 100 μM 8:2 FTOH). However, the analysis by univariate ANOVA followed by a Dunnett`s test did not corroborate the statistical significance of this reduction. The second experiment showed no difference by any sample compared to the control. In conclusion, it is possible that 6:2 FTOH and 8:2 FTOH reduced Bcrp1 expression, but the data from the experiments were inconclusive. However, the inhibitory effect on gene expression was only seen at 10 μM and 100 μM, but not at 300 μM. This lack of an increase of the effect with increasing exposure concentrations might indicate that the differences found were not due to a biologically response but rather due to variability of the method combined with relatively large technical errors.

Other authors have reported an effect on gene expression after longer exposure periods than one hour; for example Ebert *et al.* (2005) showed an induction of BCRP1 in Caco-2 cells by different flavanoids after 48 hours of treatment only. Therefore it cannot be concluded that the tested polyfluorinated compounds have no effect on the expression of Bcrp1 in rat testicular cells, since the effect might be manifested after prolonged exposure times or post exposure times.

Taken together, the presented results suggest that 6:2 FTOH, 8:2 FTOH and PFOA have no damaging effect on adult testicular cells from rats after one hour of exposure to concentrations up to 300 μM. Nonetheless, an effect on effluent transporters cannot be excluded, especially due to the short exposure time. The typical level of PFOA in human blood samples is 5 ppb which is equivalent to 0.01 μM, so the concentrations of PFOA tested here are much higher

than the normal exposure. Assuming that an exposure to 300 μM represents a no observed adverse effect level (NOAEL), the margin of exposure (MOE) is calculated to be 30,000. This is in compliance with the risk assessment done by Butenhoff *et al.* (2004a). One would normally consider a factor of > 100 to be safe with respect to non-carcinogenic effects, while a factor > 10,000 is considered safe for carcinogenic effects. To sum up, the MOE seems to be sufficient to assume that PFOA does not lead to testicular toxicity in the general population. So far the FTOHs have not been measured in human blood due to technical difficulties. It is therefore not possible to estimate human exposure based on direct measurements, making it impossible to calculate a MOE for these substances.

4.3 1,2-dibromo-3-chloropropane (DBCP)

Earlier studies have shown that DBCP can act as a mutagen and clastogen (Rosenkranz, 1975) and induces DNA damage in testicular cells (Brunborg *et al.*, 1988). The effect of DBCP regarding cytotoxicity and induction of DNA damage in adult testicular cells, spermatogonia, and somatic cells was evaluated. In addition, in the present study the repair capacity of spermatogonia and adult testicular cells was evaluated.

Exposures with up to 100 μM DBCP for one hour did not decrease viability of spermatogonia. One of the three experiments where adult testicular cells were exposed to DBCP showed a reduction in viability at 10 μM DBCP. However, this result was of borderline significance (p=0.049). In the groups treated with 30 μM DBCP, no reduction in viability was found. These results suggest no cytotoxicity of DBCP on testicular cells from adult rats and spermatogonia at the concentrations tested after one hour of exposure. Omnichinski *et al.* (1988b) found no cytotoxic effect of DBCP on testicular cells after *in vitro* exposure to 1 mM, while severe cytotoxicity was observed at 2.5 mM. These findings are in compliance with the results in our study.

DBCP was found to induce DNA damage in all testicular cell types. This is consistent with findings of others, including Brunborg *et al.* (1988) and Labaj *et al.* (2005) ,as well as studies showing that DBCP can act as a mutagen and clastogen (Rosenkranz, 1975).

The increase in %tail-DNA without the Fpg-enzyme represents single strand breaks or alkalilabile sites. Direct induction of strand breaks is not expected to occur following DBCP exposure, while AP sites can be due to unstable DNA adduct formation. This seems to be a reasonable explanation since several of the metabolites of DBCP can form adducts with DNA. as shown by Humphreys *et al.* (1991), who suggested the formation of DBCP adducts at the N^7-position of guanine. As mentioned earlier, adducts can be stable or unstable, but mainly unstable DNA adducts are measured in the comet assay (as AP sites). However, adducts that are stable under physiological conditions, can be unstable and lead to AP sites at the high pH used in the comet method. The formation of adducts at the N^7-position is expected to lead to unstable adducts under physiological conditions (Cavalieri *et al.*, 2000). The addition of Fpg in the comet assay led to an increase of %tail-DNA compared to cells not treated with Fpg. This suggests that DBCP also induces 8-oxoG, ring-opened purines or small adducts. However, the amount of Fpg-sensitive sites was far less than damage observed without Fpg treatment. It seems therefore that DBCP induces mainly abasic sites as a secondary product of adduct formation, plus some Fpg-sensitive sites.

One part of this study was to evaluate the susceptibilities of different testicular cell types. With respect to DNA damage such differences in different cells can have several explanations:

- Differences in uptake efficiency of the test compound,
- differences in metabolism and thereby bioactivation of the parent compound or
- differences in repair capacity of the induced damage.

During *in vivo* treatment the exposure of cells will differ depending on where they are situated in relation to the blood-testis-barrier (BTB), with spermatogonia and Sertoli cells receiving higher exposures than spermatocytes, spermatids and sperm cells. There are, however, no data available suggesting a difference in uptake in the three cell types *in vitro*. As described earlier, DBCP is bioactivated to reactive episulfonium ions by GST in the testis. The amount of GST has been shown to increase during spermatogenic cell development (Grosshans and Calvin, 1985); this will most likely lead to a higher bioactivation rate in later stages of spermatogenesis and thereby higher levels of DNA damage. The repair capacity is most likely reduced in spermatids compared to spermatogonia, as discussed in more detail below. These differences in repair rate can contribute to differences in observed damage levels. During the exposure period an equilibrium will eventually become established between formation of new damage induced by DBCP, and repair of these lesions. DNA damage caused by DBCP in testicular cells has been shown to be induced quite rapidly with a measurable increase as early as 10 minutes after the start of *in vitro* exposure (Brunborg *et al.*, 1988). However, in the same study it was shown that the amount of DNA damage increased throughout 60 minutes of exposure, suggesting that the equilibrium between newly induced lesions and repair was not reached during this period. It can therefore be assumed that differences in DNA damage induced in the different cell types in this study are, to a small part but not mainly, caused by different repair capacities.

To sum up, the differences in the level of DNA damage is most likely due to differences in bioactivation of DBCP, with distinctions in repair rate contributing to a lesser part in this study.

As described earlier, three different cell types were isolated: 1) testicular cells from adult rats, containing mainly spermatids but also spermatocytes and somatic cells, 2) cells obtained from prepubertal rats and incubated for one hour in lectin coated dishes, consisting of mainly spermatogonia (82%), and 3) cells from prepubertal rats incubated for 24 hours, consisting of near equal amounts of spermatogonia and somatic cells. We observed a difference in susceptibility towards DNA damage in the different cell types: The highest level of DNA damage was observed in the suspension consisting of spermatogonia and Sertoli

cells, which suggests that Sertoli cells are highly susceptible to DNA damage caused by DBCP. This is in accordance with the high levels of GST and GSH in Sertoli cells reported by Bauche *et al*. (1994). However, Bjørge *et al.* (1995) reported a higher susceptibility towards DNA damage caused by DBCP in round spermatids than in Sertoli cells. Our findings suggest a higher susceptibility of Sertoli cells. Yet, there are some confounding factors in these experiments: The somatic cells were cultured 24 hours on lectin dishes prior to exposure. The prolonged incubation of these primary cells can lead to higher levels of DNA damage due to suboptimal cultivation conditions, which itself can bring about damage to the DNA. A somewhat higher level of DNA damage observed in the control cells supports this hypothesis. Another reason for higher levels of DNA damage can be higher levels of GSH or GST in cells incubated for 24 hours after isolation. This would lead to higher bioactivation ratios of DBCP to the reactive episulfonium ion and thereby higher DNA damage levels. Freshly isolated cells can potentially have a depleted GSH or GST depot, resulting in lower bioactivation of DBCP. This theory is supported by observations made previously in our laboratory (Brunborg, personal communication). Whether the prolonged incubation time causes the increased level of DNA damage could be tested by also incubating the other two cell samples for 24 hours prior to exposure.

DBCP induced different levels of DNA damage in spermatogonia and spermatids, but the difference in response was not very distinct. However, the available data showed a trend towards less DNA damage induced in spermatogonia, as well as faster repair in these cells after exposure to DBCP. Due to the higher levels of GST in spermatids compared to spermatogonia reported by Grosshans and Calvin (1985), a higher bioactivation rate in spermatids and therefore also higher levels of DNA damage were expected in spermatids, which is in compliance with our findings. Meistrich *et al.* (2003) found seminiferous tubule of DBCP exposed rats containing no differentiating germ cells, but proliferating and dividing type A spermatogonia. These spermatogonia did not undergo differentiation, but underwent apoptosis, thus suggesting that the oligospermia is not due to a loss of stem cells, but due to a block in differentiation (Meistrich *et al.*, 2003). This block could be due to DNA damage in the stem cells, leading to an arrest of DNA replication and thereby cell proliferation.

Our results suggest therefore the following sensitivity towards DNA damage induced by DBCP: Sertoli and other somatic cells > spermatids > spermatogonia.

Biava *et al.* (1978) found seminiferous tubules from exposed individuals to be lacking germ cells, and concluded that spermatogonia and later stages of germ cells were more prone to the effect of DBCP. Such findings in humans are not reflected in our study. Amann and Berndtson (1986) found a reduction in spermatocytes/Sertoli cell-ratio, which also was

interpreted as evidence towards the higher susceptibility of germ cells. However, if Sertoli cells are severely damaged they might not be able to support the differentiating germ cells, which could lead to apoptosis of germ cells and thereby seminiferous tubuli lacking these cell types. This is supported by findings of Zhang *et al.* (2007) who showed that radiation, which leads to infertility due to azoospermia, causes damage in the supporting tissue, and not the germ cells/spermatogonia. It is possible that the same mechanisms are causing infertility after DBCP exposure, with Sertoli cells being more severely damaged then the spermatids and spermatogonia.

Taken together the results suggest a clear DNA damaging effect of DBCP to all testicular cell populations studied. Possible consequences are cellular dysfunction, cell death, or mutations. Mutations in germ cells are especially serious since they can impair fertility, increase abortion rate or lead to genetic disorders in offspring. Mutation occurring in germ cells during childhood have a greater likelihood of being transferred to offspring since germ line stem cells (spermatogonia) in children will have more opportunity to produce mutant mature germ cells than mutant germ line stem cells formed in adults (Xu *et al.*, 2008). Sertoli cells might be more susceptible towards to DBCP toxicity than spermatids and spermatogonia. The damaging effects on Sertoli cells can disturb or inhibit spermatogenesis and thereby reduce fertility or lead to infertility. This effect can be due to an impairment of the ability to provide nutritional and other supportive functions for the developing germ cells. In addition, Sertoli cells have an important role in the blood-testis barrier by maintaining tight junctions and transporting xenobiotics out of the adluminal compartment. In addition, the BTB protects germ cells from autoimmunity, a disturbance of the BTB can therefore induce autoimmunity. A disturbance of Sertoli cells can therefore lead to less protection of maturing germ cells, leading to increased chemical insults. Damage to Sertoli cells can therefore have an indirect effect on toxicity of xenobiotics towards germ cells. However, these results must be interpreted with caution as the effect of the longer preincubation time is not known.

4.3.1 Repair capacity

Whether the induced DNA damage in testicular cells has an effect on fertility and induction of cancer development depends both on the amount and types of damage induced, but also on the repair of the damage. Because of this, the efficiency to repair DNA damage induced by DBCP was evaluated in spermatogonia and spermatids.

The experiments examining repair capacity were in this study carried out in an incubation chamber containing only 5% O_2. Inside the testicle, testicular cells are exposed to approximately the same oxygen concentration, and previous experiments in our lab and other

labs have shown that primary testicular cells have reduced viability and increased DNA damage when incubated for extended time periods at 20% O_2 (Brunborg, personal communication, (Erkkilä *et al.*, 1999)).

There was no reduction in total %tail-DNA in cells treated with Fpg during 24 hours of incubation, so the amount of net Fpg-sensitive sites was not significantly changed during 24 hours incubation after DBCP exposure (appendix 7.5.5). This is likely due to the cells being exposed to reactive oxygen species during incubation and due to oxidative damage caused by handling of cells. This is in compliance with results by Torbergsen and Collins (2000) who found an increase in DNA damage in primary cultured lymphocytes during the first few hours after isolation. This suggests that the testicular cells were able to repair some of the oxidative damage, but about equal amount of new damage were induced simultaneously.

In cells not treated with Fpg, a reduction of DNA damage was observed, indicating repair of single strand breaks and alkalilabile sites in both spermatogonia and round spermatids. The reduction in %tail-DNA can possibly be due apoptosis and following disintegration of heavily damaged cells, leading to a selection of cells with little DNA damage. However, cell viability as determined by the Trypan Blue Exclusion test showed viability >88% in all samples, so this seems an unlikely source of error. To ensure that apoptosis is not contributing to a sample error the number of cells in each sample should be monitored. Another possibility would be to add an enzyme that inhibits DNA repair to a parallel set of exposed cells. If a decrease in %tail-DNA would be observed in these samples as well, selection of cells rather than DNA repair would be the explanation. Bjørge *et al.* (1997b) report a repair of DNA damage induced by DBCP in spermatocytes, but not in spermatids, which is in contrast to our findings.

The observed $t_{1/2}$ in this *in vitro* study is likely to be longer than the actual repair rate *in vivo* because the cells in suspension lack the support from Sertoli cells (nutrition, growth factors etc). This hypothesis is supported by findings of Bentley and Working (1988b; , 1988a) who observed 4-25 times higher repair rates in germ cells exposed *in situ* in seminiferious tubules than in germ cells exposed in suspension.

A slight difference in the repair rate of spermatogonia and spermatids was observed. The results presented suggest a somewhat faster repair of DNA damage in spermatogonia than in spermatids. This is in compliance with findings by Matulis and Handel (2006) who found little repair in later stages of spermatogenesis. As described earlier, it has been suggested that BER is functional in all germ cell stages while NER is reduced in spermatocytes more or less absent in round spermatids (Jansen *et al.*, 2001; Olsen *et al.*, 2001; Xu *et al.*, 2005; Matulis

and Handel, 2006). Small adducts, as are expected after DBCP exposure, are likely removed by both NER and BER. This is in compliance with our findings of active repair of DBCP induced lesions in both cells types with a higher repair rate in spermatogonia.

Biologically, the reduced repair capacity in later stages of spermatogenesis might make sense, as these cells are somewhat protected by the blood-testis barrier from harmful components. Spermatogonia, however, are needed as a stock to provide germ cells during the reproductive phase of males, so effective and functional repair systems in these cells are highly meaningful.

5. Conclusions and future work

The results obtained in this study suggest that 6:2 FTOH, 8:2 FTOH and PFOA have no severe detrimental effect on testicular cells from adult rats, and are not likely to have a negative impact on male fertility. However, none of the data have conclusive value due to few experiments with short exposure times and statistical tests with low observed power. Furthermore, the comet assay measures single strand breaks, abasic sites, oxidized guanine, and possibly some small adducts. We can therefore not deduce that the tested PFCs are not genotoxic, since other DNA lesions as bulky adducts are not measured in this assay. In addition, the actual exposure was not measured since the concentration throughout the exposure time was not monitored. Therefore longer exposure or post exposure times must be examined, both regarding cytotoxicity, induction of DNA damage and alteration in gene expression, together with control of concentrations during exposure. The longer incubation times might best be accomplished using *in situ* exposure of cells in seminiferous tubules. The expression of P-gp could be studied either by real-time PCR or with flow cytometric analysis of cells stained with anti-P-gp after prolonged exposure. Alternative methods are Western analyses or measurement of ATPase activity. Also, the potential enzyme inhibition by PFOA could be analysed with a modified comet assay as described earlier. To positively exclude genotoxicity, the compounds have to be tested in other genotoxicity assays as well.

Taken together with the data described in the literature study, the main effects of the polyfluorinated compounds in rodents seem to be an induction of peroxisomal proliferation, a detrimental effect on reproduction after *in utero* exposure, and a possible effect on hormone homeostasis and chemosensitization. The testicular toxicity, however, appears to be low. Whether human health is influenced by these substances remains inconclusive. The NOAEL values obtained in both *in vitro* and *in vivo* experiments suggest a high MOE for PFOA. No such value can be calculated for the FTOHs since no data exist on blood levels or exposure of these substances. Whether there actually is a risk posed to humans depends not only on the MOE-value, since the mode of action can be quite different in humans and rodents, which is particularly true for peroxisomal proliferation.

DBCP is a known testicular toxicant in both humans and rats, but the mechanism of toxicity still remains somewhat unclear. The main mode of action appears to be induction of DNA damage leading to an arrest of differentiation of germ cells. The type of cells most affected is however still uncertain. In order to evaluate the induced DNA damage in different cell types, a method for isolation of different cell populations was needed. The isolation of testicular

cells from prepubertal rats, followed by one hour of incubation on lectin coated dishes, resulted in a cell population consisting of approximately 80% spermatogonia. The method is convenient and combined with the high purity of the appropriate cell types it seems to be a well fit method for isolation of spermatogonia. In this study, DBCP was shown to induce DNA damage in all cell types tested and a trend towards the following sensitivity was observed: Sertoli cells and other somatic cells > round spermatids > spermatogonia. The high susceptibility of Sertoli and other somatic cells observed can, however, be due to prolonged incubation prior to exposure. This needs to be examined further. The repair of the DNA damage seems to be higher in spermatogonia compared to round spermatids, but the results presented here are only preliminary, since they are based on only one experiment. More experiments are needed to strengthen the possible differences between cell types. Later on, it would be interesting to study the repair capacity in Sertoli cells as well.

Taken together, the results suggest that DBCP can impair male fertility by damaging the DNA of several testicular cell types. In contrast, the selected polyfluorinated compounds showed no clear testicular toxicity. Nonetheless, this is no proof of a lack of toxicity, or as Albert Einstein put it: “No amount of experimentation can ever prove me right; a single experiment can prove me wrong”.

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7. Appendix

7.1 Chemicals

Chemical	**Producer**
Dubeccos`s Phosphate Buffer solution (PBS)	Locally produced
Dimetyhylsulfoxide (DMSO)	Merck, Germany
Bovine serum, albumin (BSA)	Sigma, USA
Bio Whittaker® RPMI 1640 medium with 25mM Hepes and L-Glutamin	Lonza, Belgium
Fetal calve serum (FCS)	Gibco, NY, USA
Sodium Pyruvate	Sigma, Japan
DL-Lactic acid	Sigma, Norway
P/S	Sigma, Norway
Collagenase type 2, 275 U/mg	Worthington, USA
Trypsin, 10200 U/mg	Sigma, USA
Lectin from *Datur stramomium*	Sigma, USA
Sodium chloride (NaCl)	Merck, Germany
Sodium Hydroxide (NaOH)	Merck, Germany
Hydrogen chloride (HCl)	Merck, Germany
Potassium hydroxide (KOH)	Merck, Germany
Potassium chloride (KCl)	Merck, Germany
Trizma® base (Tris (hydroxymethyl)-aminomethane, Tris-base)	Sigma, USA
Triton-X	Sigma, USA
Sodium lauryl sarcocinate	Sigma, UK
GelBond® Film	Cambrex, USA
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma, USA
Hepes	Sigma, USA
NuSieve GTG Low melting agarose	Cambrex, USA
Fpg crude enzyme extract	Locally produced
SYBR Gold	Invitrogen, USA
Perfluorooctanoic acid (PFOA)	Wellington, Canada
1H,1H,2H,2H-perfluoro-1-decanol (8:2 FTOH)	Sigma-Aldrich, Japan
1H,1H,2H,2H-Perfluoro-1-octanol (6:2 FTOH)	Sigma-Aldrich, USA
1,2-dibromo-3-chloropropane (DBCP)	Dr. Nelson, USA
SV Total RNA Isolation System, Cat #AZ3105	Promega, USA
Reverse Transcription System, Cat # A3500	Promega, USA
Bcrp primer (rBcrp1F and rBcrp1R)	Sigma
18S primer (H18S-F and H18S-R)	Sigma
Oat2 primer, Slc22a7	SuperArray
Power SYBR Green PCR Master Mix	Applied Biosystems, UK
Distilled water, DNase and RNase free	Gibco, USA
Vimentin Clone V9, Mouse, Code No M0725	Dako AS, Denmark
Secondary antibody, RRX anti-mouse	Jackson Immuno Research, West Grove,

	PA, USA
Paraformaldehyd (PFA)	Merck, Germany
Hoechst 33258	Calbiochem-Boehringer, USA
Thimerosal	Sigma, USA
Trypan Blue Stain	Cambrex, USA
Polysine Microscope slides	Menzel, Germany
Absolute alcohol prima	Arcus Kjemi, Norway
Micro Amp 96-Well Reaction Plate	Applied Biosystems, Singapore

7.2 Solutions and media

Media

Testis medium with serum: RPMI 1640 added 10% FCS, 0,1 mg pyruvat/ml, 5mM DL-Lactic acid, and 1% P/S

Testis medium without serum: RPMI 1640 added 0,1 mg pyruvat/ml, 5mM DL-Lactic acid, and 1% P/S

Solutions used in the comet assay

Lysis stock solution:
2,5 M NaCl, 100 mM EDTA, 10mM Trizma base, 12g/l NaOH. Adjust pH to 10. Add 1% Sodium lauryl sarcocinate.

Lysis solution:
Dilute stock solution 10 times, add 10% DMSO and 1% Triton-X

Fpg-enzyme reaction buffer:
40mM Hepes, 0,1 M KCl, 0,5mM EDTA. pH adjusted to 7,6.

Unwinding and electrophoresis stock solution:
10N NaOH, 200mM EDTA

Unwinding and electrophoresis buffer:
Dilute electrophoresis stock solution 10 times, adjust pH to 13,2

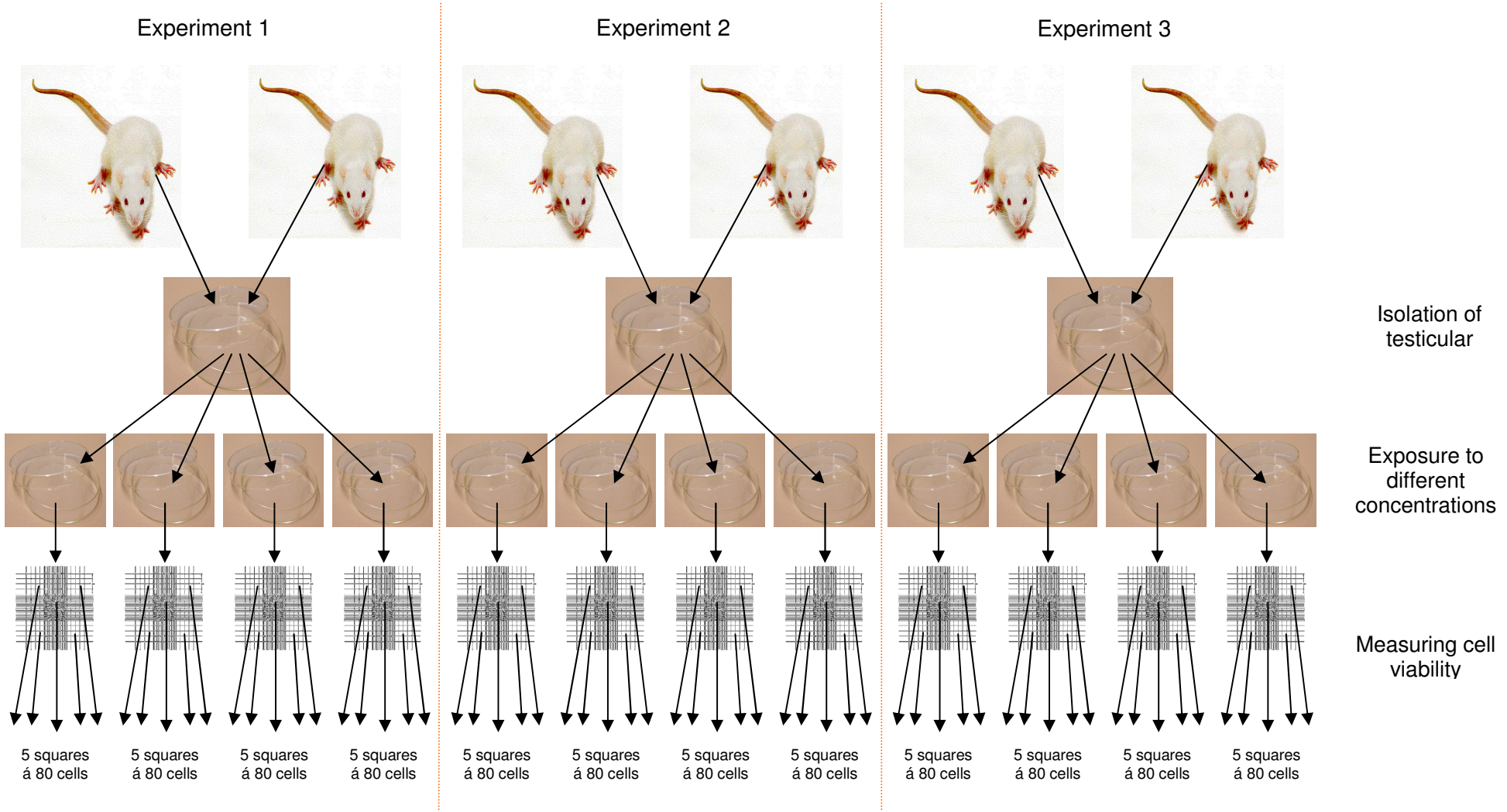
Neutralization solution:
0,4 M Trizma base, pH adjusted to 7,5.

TE-buffer:
1mM EDTA, 10mM Tris HCl, pH adjusted to 8,0.

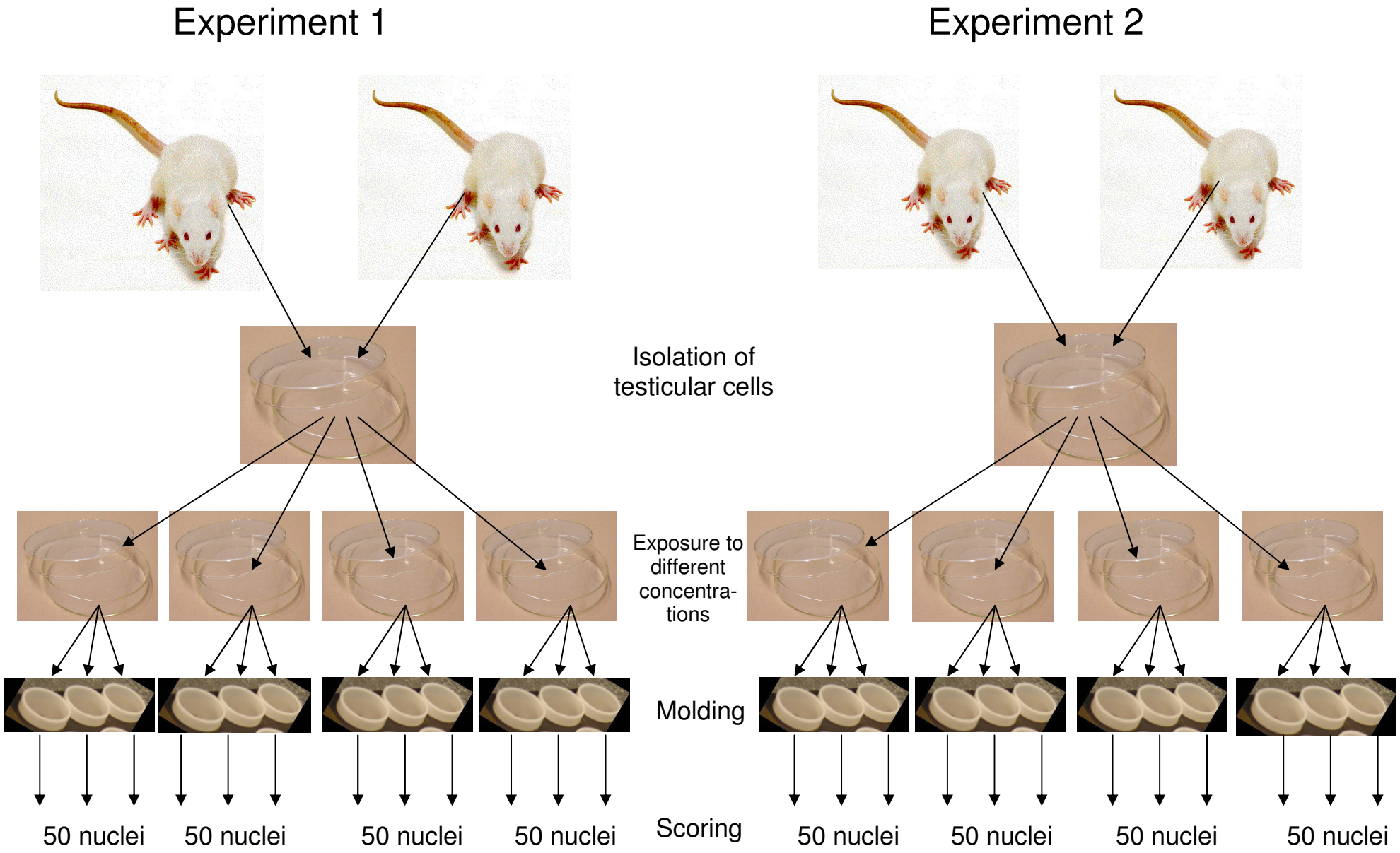
7.3 Experimental design

7.3.1 Cytotoxicity
7.3.2 Comet assay
7.3.3 Gene expression
7.3.4 Repair capacity

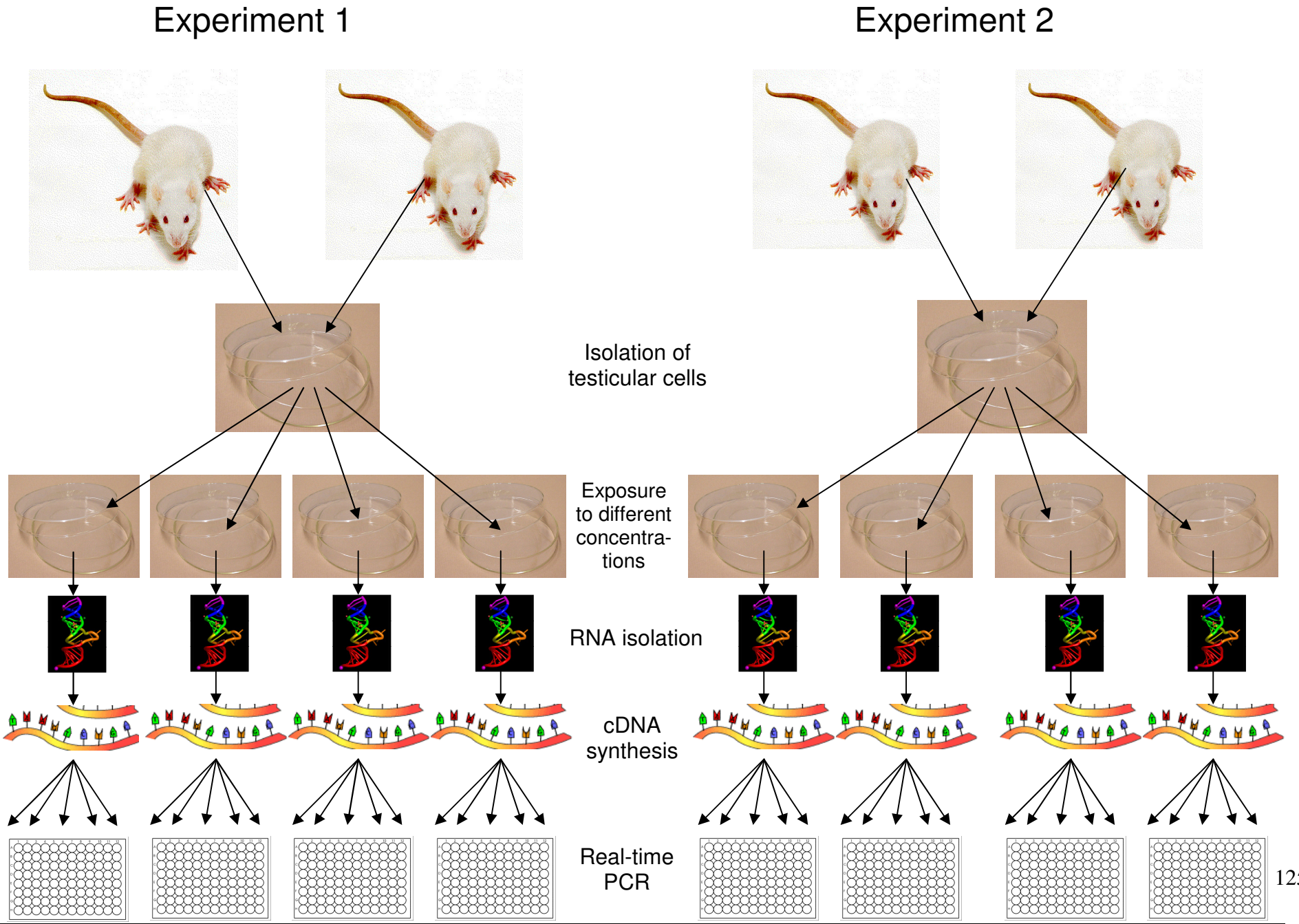
7.3.1 Experimental design – Cytotoxicity



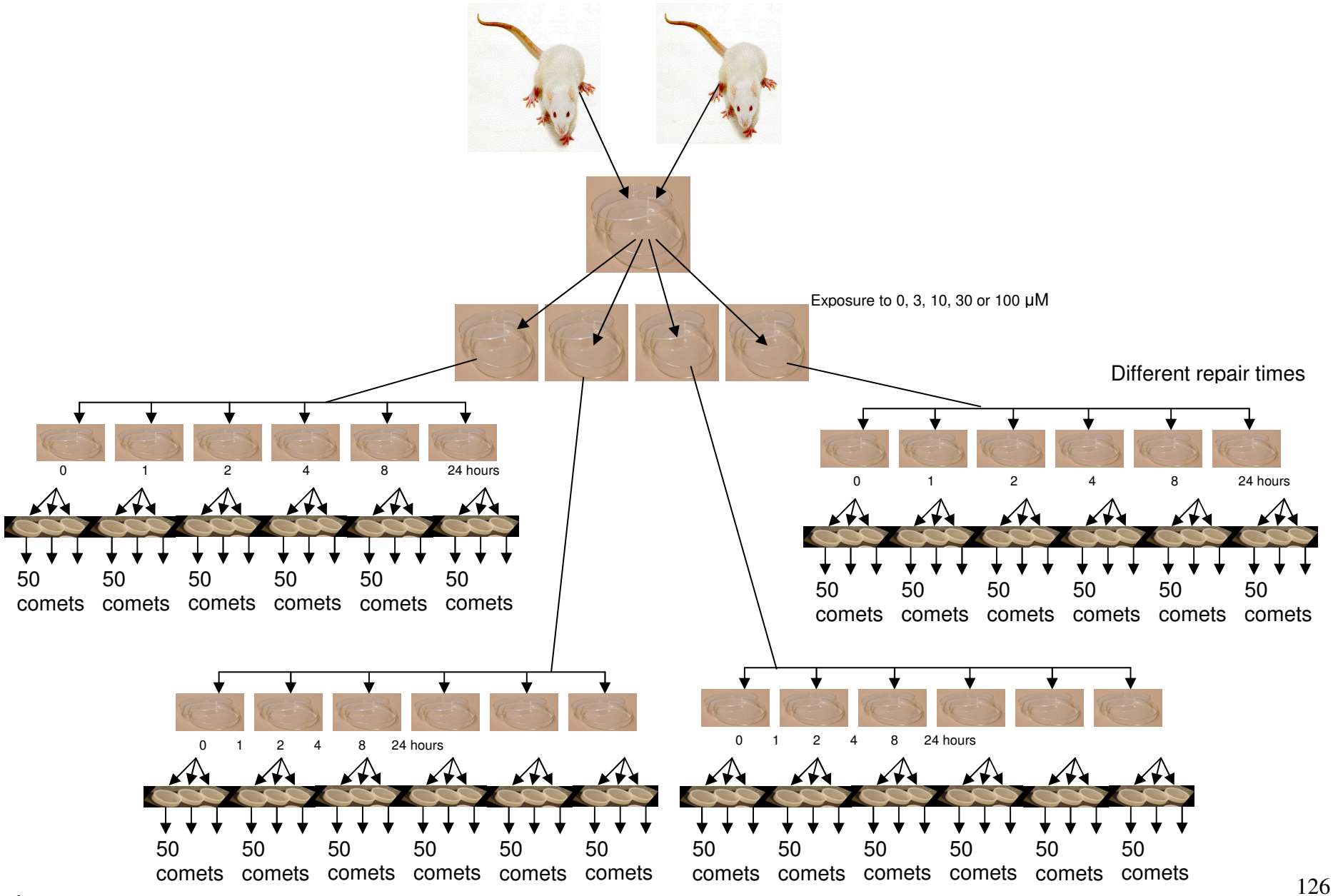
7.3.2 Experimental design – Comet assay



7.3.3 Experimental design – Gene expression



7.3.4 Experimental design – DNA repair capacity

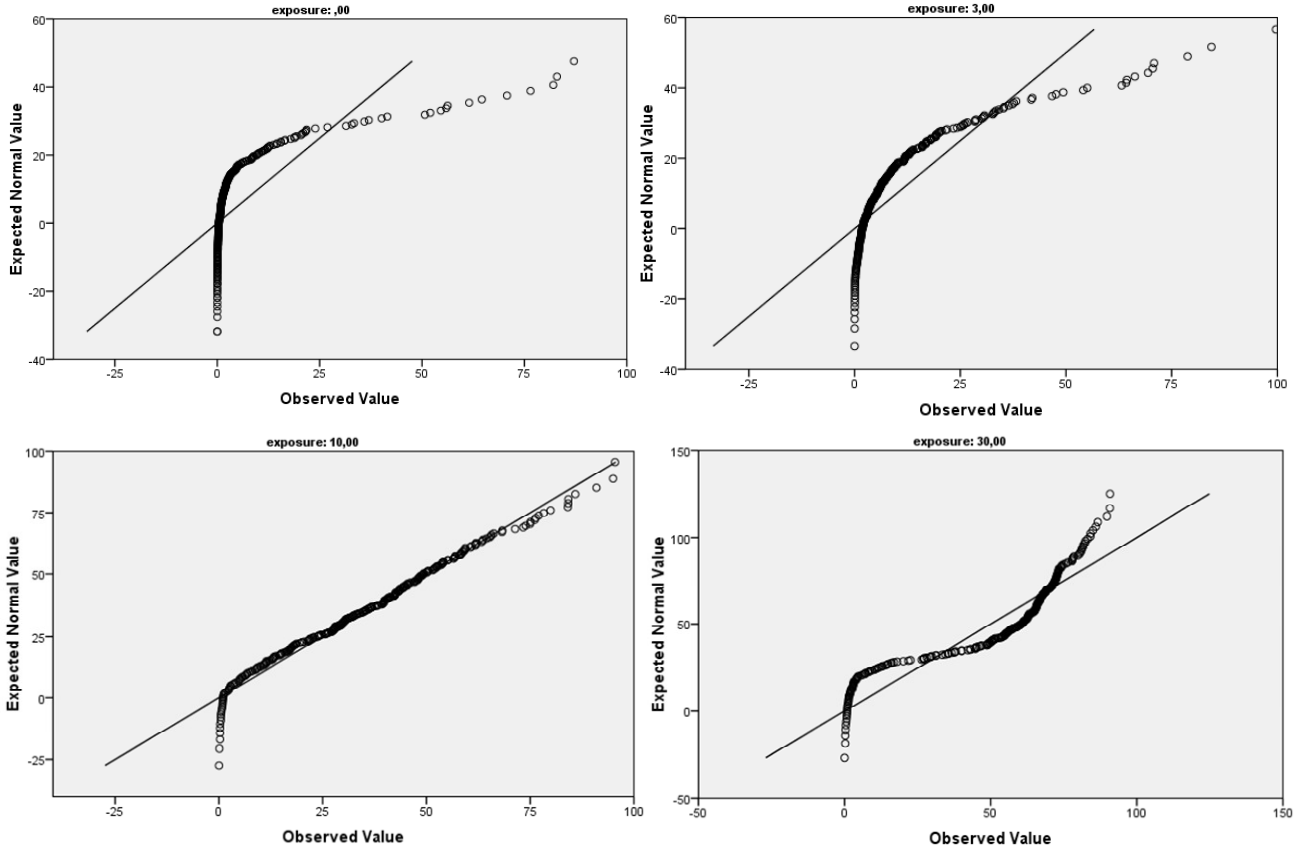


7.4 Table of test chemicals

Common name	Abbreviation	Chemical name	Chemical structure	CAS number
Dibromochloropropane	**DBCP**	**1,2-dibromo-3-chloropropane**	**$CH_2BrCHBrCH_2Cl$**	**96-12-8**
Perfluorooctanoic acid	**PFOA**	**Perfluoro-n-octanoic acid**	**$C_8HF_{15}O_2$**	**335-67-1**
8:2 fluorotelomeralcohol	**8:2 FTOH**	**3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluoro-1-decanol**	**$CF_3(CF_2)_7CH_2CH_2OH$**	**678-39-7**
6:2 fluorotelomeralcohol	**6:2 FTOH**	**3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-1-octanol**	**$CF_3(CF_2)_5CH_2CH_2OH$**	**647-42-7**

7.5 Additional results

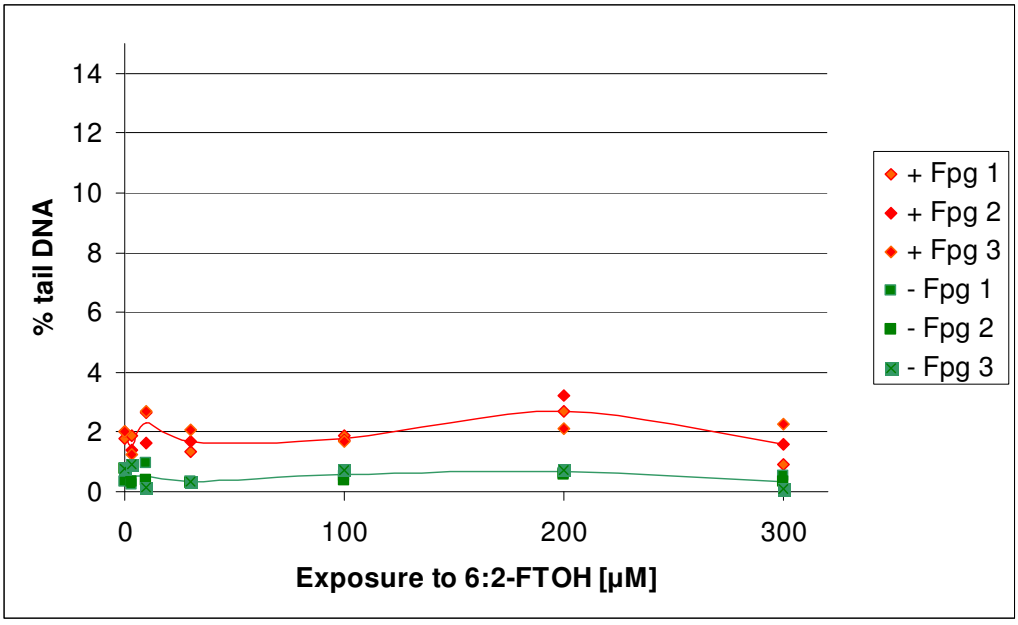
7.5.1 QQ-plots of data from the comet assay

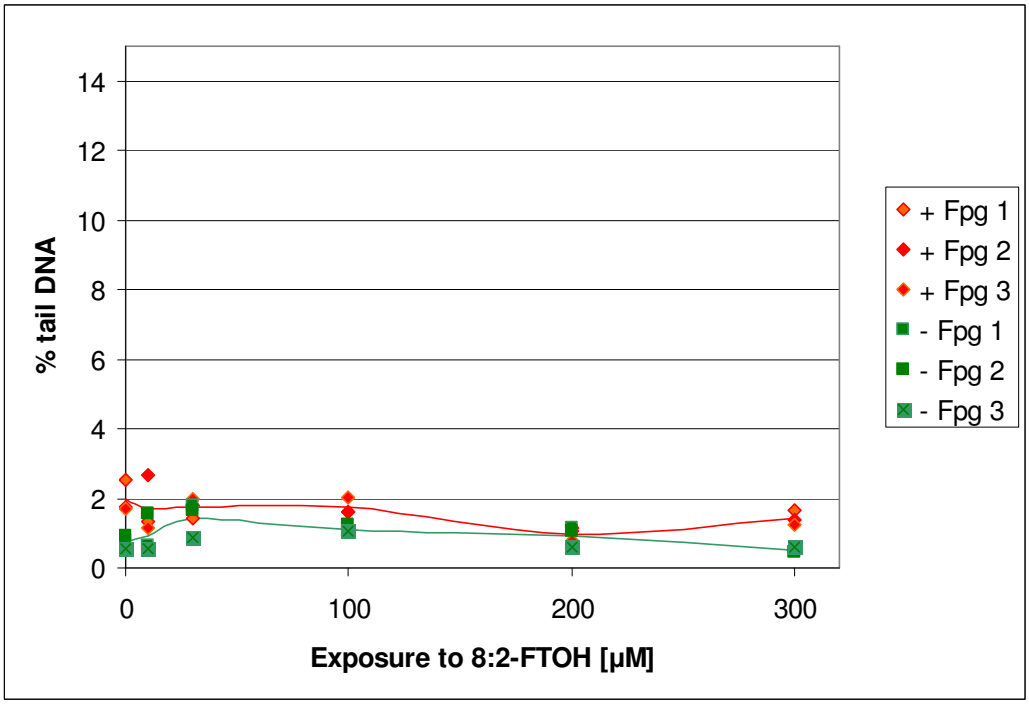


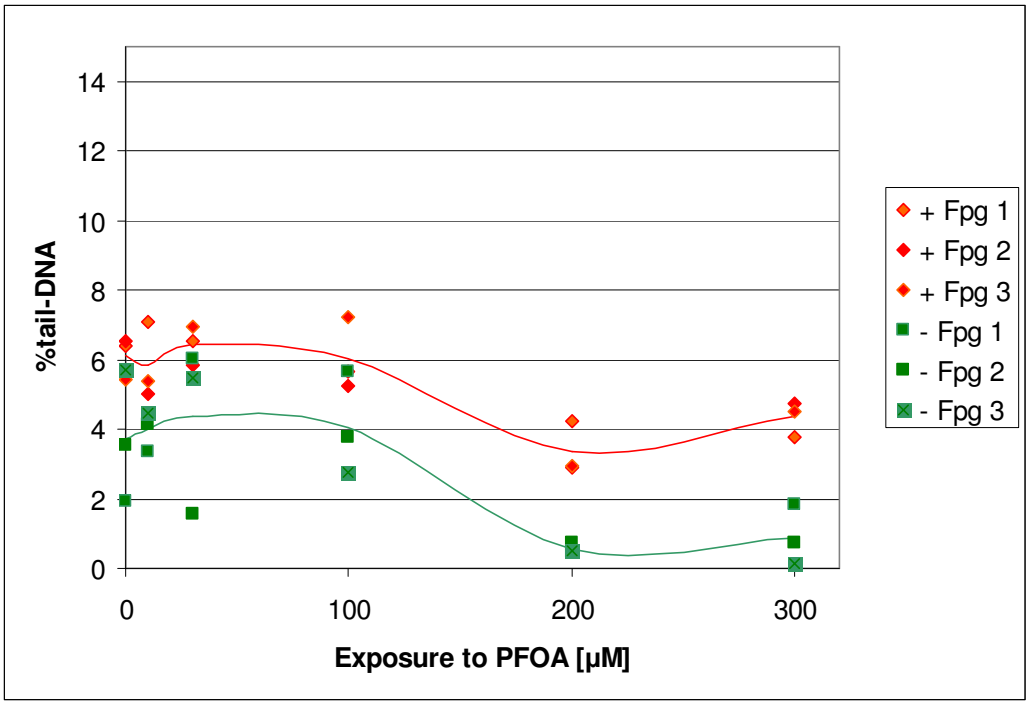
QQ-plots for %tailDNA from cells treated with 0 µM DBCP (top left), 3 µM DBCP (top right), 10 µM (bottom left) and 30 µM DBCP (bottom right).

7.5.2 Comet results from PFCs, with and without Fpg

Comet data for testicular cells exposed to 6:2 FTOH, 8:2 FTOH or PFOA.
Red dots represent median values from each gel treated with Fpg, while green dots represent median values from each gel without Fpg. The lines represent the mean value of the medians.






14
12
10
8
6
4
2
0
%tail-DNA
0
100
200
300
Exposure to PFOA [µM]
+ Fpg 1
+ Fpg 2
+ Fpg 3
- Fpg 1
- Fpg 2
- Fpg 3

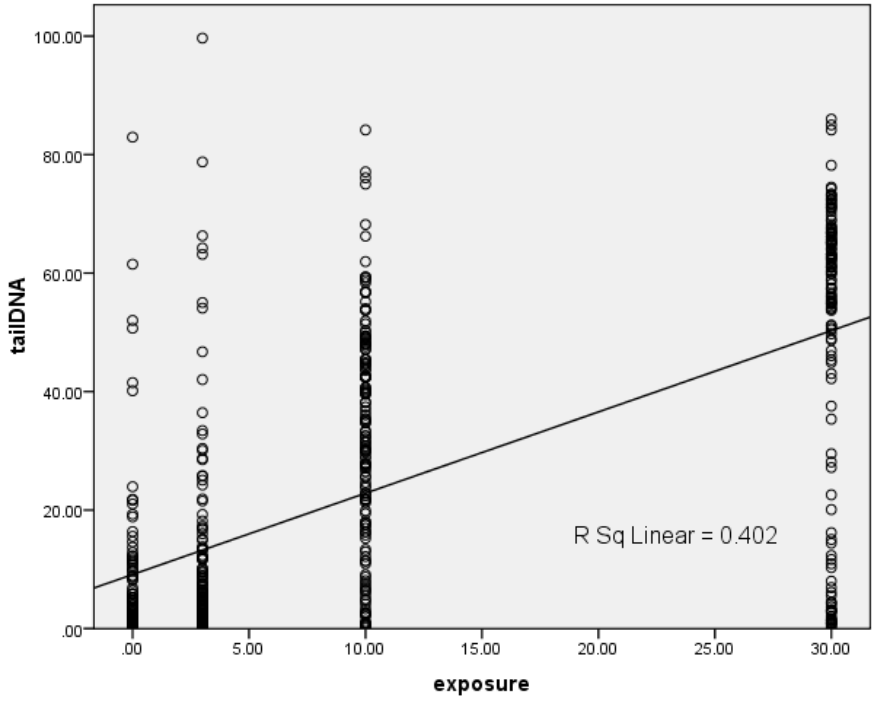
7.5.3 Testing of logtransformation of Comet data

Data obtained in the comet assay were analyzed regarding their normality and homoscedasticity. In addition, p-values from the univariate ANOVA of the nested data together with F-values are shown. The data were also logtransformed, and the same analysis was carried out with the logtransformed data.

Results of the statistical analysis testing whether logtransformation of Comet data alters results. Shown are p-values for normality (p<0.05 meaning that the data are not normally distributed), p-values for homogeneity of variance (p<0.05 meaning that the data do not have equal variance), F-value for univariate ANOVA and p-values from the univariate ANOVA. Asterisks indicate significance.

	Parameter	Raw data	Logtransformed data
Spermatogonia exposed to DBCP, gels treated with Fpg	Normality	<0.001 *	<0.001 *
	Homogeneity of variance	<0.001 *	<0.001 *
	F-value from ANOVA	90.652	85.247
	p-value from ANOVA	<0.001 *	<0.001 *
Adult testicular cells exposed to DBCP, gels treated with Fpg	Normality	<0.001 *	<0.001 *
	Homogeneity of variance	<0.001 *	<0.001 *
	F-value from ANOVA	86.849	68.950
	p-value from ANOVA	<0.001 *	<0.001 *
Adult testicular cells exposed to 8:2 FTOH, gels treated with Fpg	Normality	<0.001 *	<0.001 *
	Homogeneity of variance	0.025 *	<0.001 *
	F-value from ANOVA	1.221	1.493
	p-value from ANOVA	0.549	0.382
Adult testicular cells exposed to PFOA, gels treated with Fpg	Normality	<0.001 *	<0.001 *
	Homogeneity of variance	<0.001*	<0.001 *
	F-value from ANOVA	2.548	2.226
	p-value from ANOVA	0.329	0.216
Adult testicular cells exposed to 8:2 FTOH, gels treated with Fpg	Normality	<0.001 *	0.003 *
	Homogeneity of variance	0.001 *	<0.001 *
	F-value from ANOVA	3.480	2.777
	p-value from ANOVA	0.077	0.120

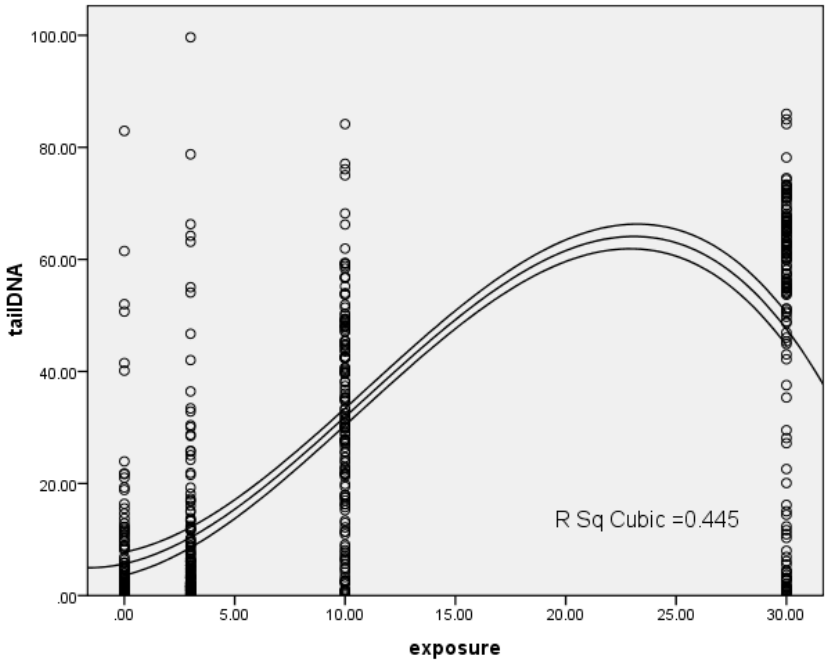
7.5.4. Dose-response models for DBCP-treatment of adult testicular cells.



Model Summary

R	R Square	Adjusted R Square	Std. Error of the Estimate
.634	.402	.401	19.600

The independent variable is eksplusslitt.



Model Summary

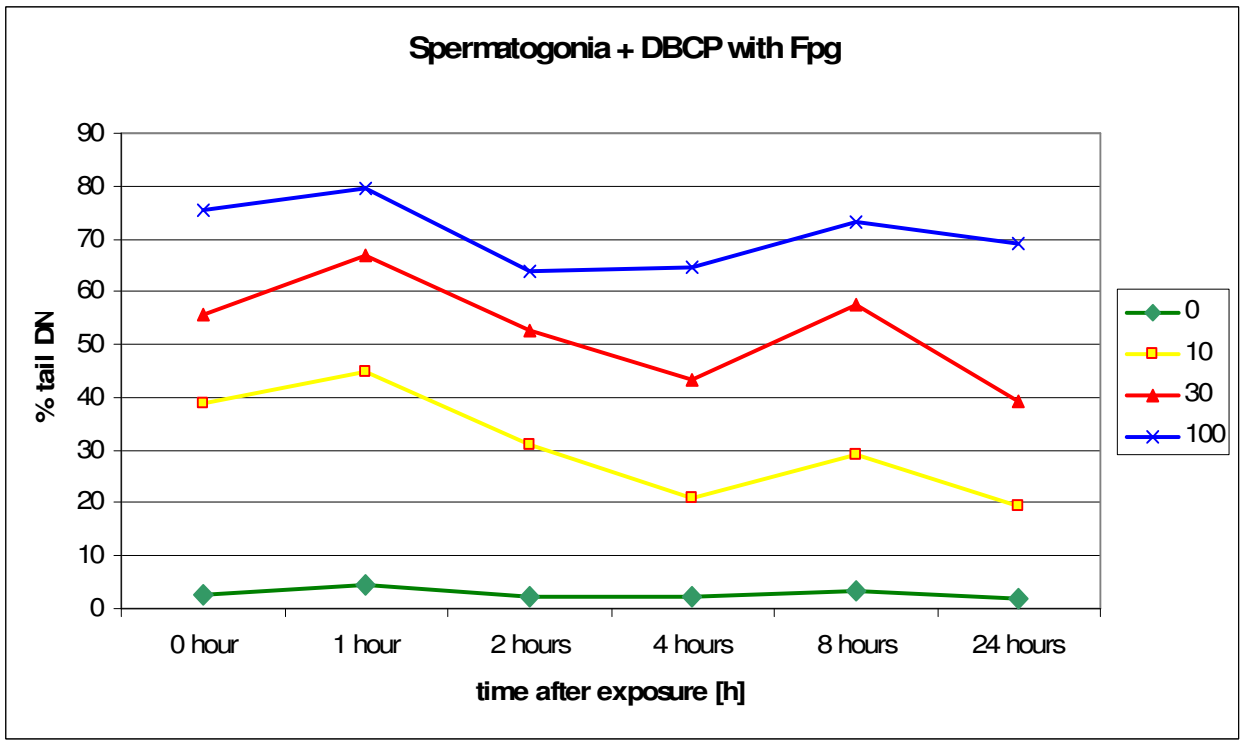
R	R Square	Adjusted R Square	Std. Error of the Estimate
.667	.445	.442	18.920

The independent variable is eksplusslitt.

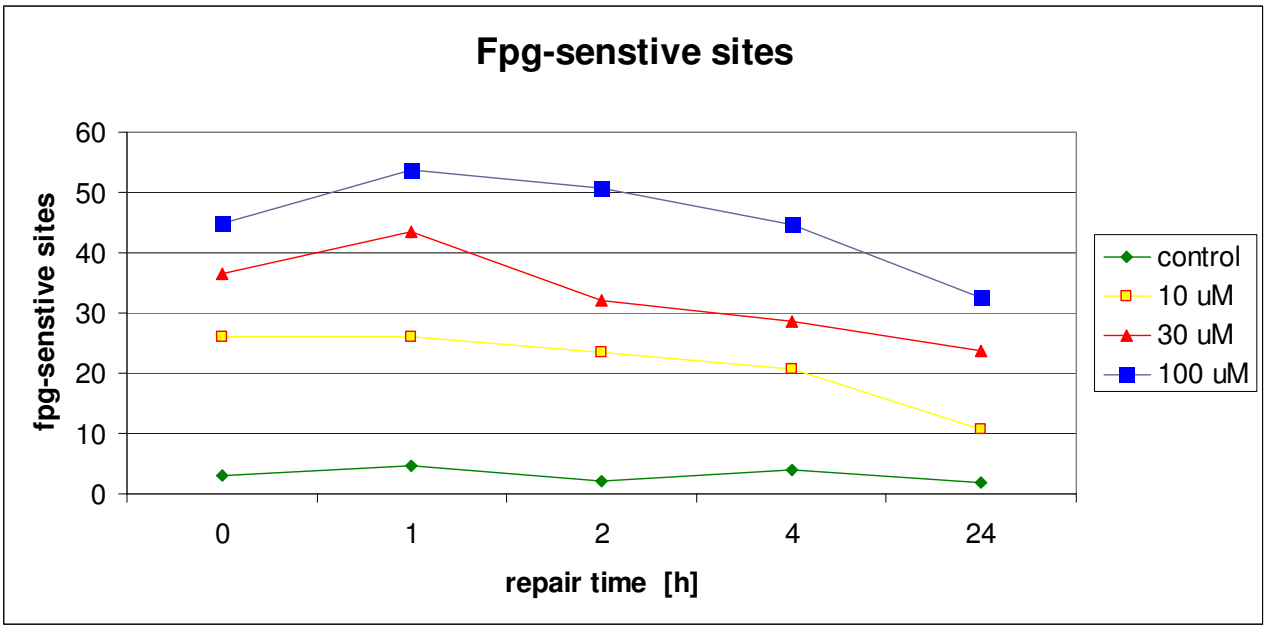
Plot of the linear model and the cubic model and the coefficient of determination for the models, together with a model summary.

7.5.5 Results from the DNA repair capacity analysis, results from cells treated with Fpg

DNA damage measured in spermatogonia after exposure to DBCP. Results from cells treated with Fpg are shown. No distinct reduction in DNA damage was observed.



Net Fpg-sensitive sites after DBCP exposure.



7.5.6 Results of statistical analysis of real-time PCR data – approach 2

Results of the statistical analysis of data from real-time PCR. Shown are p-values for normality (p<0.05 meaning that the data are not normally distributed), p-values for homogeneity of variance (p<0.05 meaning that the data do not have equal variance), F-value for univariate ANOVA and p-values from the univariate ANOVA. Asterisks indicate significance.

Exposure	Parameter	Raw data	Log transformed data
6:2 FTOH, 1st experiment	Normality	0.827	0.736
	Homogeneity of variance	0.100	0.135
	F-value from ANOVA	11.787	11.876
	p-value from ANOVA	<0.001 *	<0.001 *
8:2 FTOH 1st experiment	Normality	0.742	0.757
	Homogeneity of variance	0.374	0.406
	F-value from ANOVA	3.981	3.978
	p-value from ANOVA	0.027 *	0.022 *
PFOA 1st experiment	Normality	0.349	0.403
	Homogeneity of variance	0.025 *	0.037 *
	F-value from ANOVA	2.307	2.093
	p-value from ANOVA	0.116	0.141
6:2 FTOH 2nd experiment	Normality	0.270	0.192
	Homogeneity of variance	<0.001 *	<0.001 *
	F-value from ANOVA	1.78	1.77
	p-value from ANOVA	0.190	0.192
8:2 FTOH 2nd experiment	Normality	0.444	0.247
	Homogeneity of variance	0.502	0.421
	F-value from ANOVA	0.114	0.160
	p-value from ANOVA	0.950	0.921
PFOA 2nd experiment	Normality	0.922	0.884
	Homogeneity of variance	0.649	0.535
	F-value from ANOVA	1.362	1.425
	p-value from ANOVA	0.290	0.275

Results for Dunnetts test of control groups versus exposed groups for the exposures that did show significant differences in the univariate ANOVA. P-values for both raw data and log-transformed data are shown. Asterisks indicate significant results.

Testing	p-value for raw data	p-value for log-transformed data
control vs. 10 µM 6:2 FTOH	<0.001 *	<0.001 *
control vs. 100 µM 6:2 FTOH	0.001 *	0.001 *
control vs. 300 µM 6:2 FTOH	0.423	0.416
control vs. 10 µM 8:2 FTOH	0.307	0.331
control vs. 100 µM 8:2 FTOH	0.917	0.904
control vs. 300 µM 8:2 FTOH	0.193	0.178